Report on **Oil of Clove & Clove** [illegible] 1/4/73

#29-I

REPORT

ACCESSION NO. 18

OIL OF CLOVE

C.A.S. REG. NO. MX8000348

and

CLOVE

C.A.S. REG. NO. 977007796

RETURN TO:
GRAS REVIEW BRANCH
DIVISION OF PETITIONS PROCESSING
-FDA

GRAS Review Branch (BF-335)
Bureau of Foods
Food and Drug Administration
200 C Street, SW
Washington, D.C. 20204

Submitted to Att: Mr. Alan Spiher,
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Date January 4, 1973

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OIL OF CLOVE

Summary: Toxicological Information

Oral toxicity studies of clove spice or clove oil seem not to have been undertaken, however toxicity studies are reported for eugenol which is the major component of clove oil. The rat LD_{50} for pure eugenol administered orally ranges from 1930 mg/kg (166) to 2680 mg/kg (177). The major physiological features associated with the toxic condition produced in these animals included both lower jaw and hind leg paralysis. The forelimbs seemed to remain unaffected except when general prostration or coma ensued. Animals surviving the acute symptoms continued to show impaired hind leg function, lethargy, and urinary incontinence for several days. Microscopic examination of various tissues suggested that eugenol toxicity results from circulatory collapse which in turn leads to a general tissue congestion. (166)

Oral $LD_{50's}$ for mouse and guinea pig (64) are 3000 and 2150 mg/kg respectively, while for eugenol administered intraperitoneally, the mouse LD_{50} is 500 mg/kg. (20)

In dogs the oral LD_{50} was found to be approximately 500 mg/kg at which levels "vomiting frequently occurred along with marked motor dysfunction, primarily of the hind limbs". (103) Other dogs in this study were able to tolerate repeated daily oral doses of eugenol at the 200 mg/kg level (ten exposures over a three week period) without developing any of the above toxic symptoms, suggesting to these investigators the absence of any cumulative effects. (103)

However, in rats which had received but four daily doses of eugenol at 1/3 the LD_{50} value (approximately 1000 mg/kg), "mild liver lesions" were found upon autopsy of the animals. These lesions were characterized by "slight discoloration, mottling and blunting of the lobe edges"; (177) thus there is a possibility that similar histopathological changes had occurred in the dogs without becoming outwardly manifested during the observational period.

The irritant effect of ingested eugenol on the gastro-intestinal tract was determined by in vivo studies on dogs, originally carried out for the purpose of identifying an accessible, sensitive carcinogen-receptive site. (72) In initial studies of a dozen compounds tested, clove oil and eugenol were the two most effective topically applied substances in stimulating the production of gastric mucous in Heidenhain-pouch dogs. Clove oil and especially eugenol (5% emulsion) were found to stimulate the production of large volumes of alkaline secretion (71, 73) while acting as powerful desquamatory agents leading to the impairment and often to the complete destruction of the mucous barrier. Significantly, within thirty-six hours following removal of eugenol from the site, the mucosa of these animals was found to be completely and smoothly resurfaced with new squamous cells. (72, 167, 169)

In a study similar to the above, it was found that keeping any of the spices (nutmeg, sage, clove, etc.) in direct contact with the gastric mucosa for up to three hours produced "little or no change". However, an aqueous or oil suspension of cloves as well as a suspension of eugenol applied topically to various sections of the gastric mucosa, did produce certain changes which were due either to irritation of the whole mucosa or to stimulation of the mucous secreting cells. The changes associated with the former varied from edema and congestion to necrosis. (154)

Because the initial destruction of the mucous surface stimulates the production both of an alkaline gastric fluid and layers of new squamous cells, eugenol and certain eugenolates were found useful in the treatment of experimentally produced stomach ulcers. In one such study (193) acute peptic ulceration was produced with massive doses of histamine in a group of guinea pigs protected by an anti-histaminic, promethazine hydrochloride. A second group similarly stressed received in addition an oral dose of sodium, barium or calcium eugenolate which prevented or significantly reduced the severity of ulceration. The eugenolates were found to be more effective in this regard than eugenol (193) which may be due to some combination of factors, including the increased solubility of the former in the gastric fluid, the release rate of the free eugenol, and the acid-neutralizing power of the alkaline ions. While it is not clear what precise role is played by the cations, it has been established that neither calcium nor magnesium ions are involved in the desquamatory process. (93)

The desquamatory action of eugenol may be non-specific since, as observed in the albino rat, it was found to impair both gastric leucopedesis and mast cell diapedesis. The same effects are also induced by histamine dihydrochloride. (18)

Eugenol was found to have an effect on several liver enzyme systems. A 50% inhibition of o-aminophenol glucuronide by liver slices was obtained with a eugenol concentration of 2 μg/ml in the incubation solution, while at a concentration of 200 μg/mg or more the activity was reduced to less than 5% of that of the controls. Similarly in *in vivo* experiments 3 ml of 5% eugenol given to animals resulted in virtually complete inhibition of glucuronide synthesis in rinsed stomach. Histological studies of the stomach after this treatment revealed desquamation of the surface at the epithelium, (65) an effect of eugenol already reported by earlier investigators.

Although able to rapidly produce local irritation, eugenol and iso-eugenol when applied topically showed no percutaneous absorption within a two hour period when applied to the intact shaved abdominal skin of the mouse. In contrast, very rapid absorption was observed with such oils as geranyl formate, terpineol, thymene, carvone and rue oil. (119) Regardless of the absorption rate most of the essential oils produce more or less severe skin lesions when applied cutaneously for a sufficient length of time;; some are used for this purpose in studies on carcinogenic processes. In one such study clove oil and eugenol were applied separately to the shaved dorsal skin of mice to determine whether the irritant effects under certain circumstances might lead to cancer induction or act as tumor promoting agents. Despite the fact that both these substances were extremely irritating to the animals' skin they proved to be inactive as tumor promoting agents. (146)

One result of the irritant effect of clove oil as observed in the rabbit was the acceleration and strengthening of the peristaltic movements of the animals' small intestine. This effect was the same both for the excised intestine and for the intestine in situ. (168) The reverse action was found in a study aimed at evaluating a number of essential oils including clove oil as possible anti-spasmatic agents. Each oil (as a saturated aqueous solution) was tested against such spasmogens as adrenalin, acetycholine, $BaCl_2$, histamine, and nicotine, which had been used in advance to activate the isolated seminal vesicles, duodeum, ilium, aorta and jejunum of rats, guinea pigs and rabbits. All of the solutions proved to have a "clear anti-histamine action and to inhibit nicotinic spasms". Clove oil in particular showed musculotropic spasmolytic activity but was ineffective against neurotropic spasms due to acetylcholine. (34)

Eugenol, caryophyllene, berberine, and condurangin administered s. c. or i. v. in singles doses or in successive injections into normal rabbit had no effect on erythrocyte number, leucocyte number, or on the amount of blood pigment. On the other hand, when administered to the toluene-diamine anemic rabbit, all of the compounds except berberine showed definite anti-anemic activity. (97)

Eugenol and several other members of the eugenol series including cis-isoeugenol and trans-isoeugenol, produce a prompt lowering of body temperature when injected i. p. (as a 5% solution in olive oil) into mice. On a dose basis of 0.1 gm/kg body weight, the maximum temperature depression of these compounds was : eugenol 1°C at 40 minutes; cis-isoeugenol 4°C at 30 minutes; trans-isoeugenol 1.5°C at 20 minutes. (20)

Clove Oil and eugenol show bacteriostatic and bacteriocidal activity against the bacterial species Eberthella typhosa, Monilia albicans (7) and are toxic to infusoria consisting of Paramecium caudatum and Trichomonas vaginalis (150). Both eugenol and isoeugenol were also found to be anti-helminthic agents when tested in vitro against a number of different animal parasites. They were also effective in vivo as established from tests made with the oxyures of the white mouse. (182). Eugenol exhibits some degree of local anesthetic action in addition to its antiseptic power, and because of these characteristics is frequently used by dentists for disinfecting root canals, as a local anodyne for the relief of hypersensitive dentine, of pain and irritation incident to hyperemic and inflamed vital pulps, and as a component of the zinc-eugenol cement employed as a temporary filling for carious teeth. (181a). However chemical and histological studies showed that eugenol and ZnO-eugenol mixtures progressively removed calcium from sound dentine. It is hypothesized that the softening of the dentine beneath the mixture was due to the formation of the calcium-eugenol complex involving the excess eugenol not entering into the setting matrix. (149).

CLOVE and OIL OF CLOVE

Chemical Information

I. Nomenclature (39a, 48a)

A. Common Names

Clove

Clove Oil

Oil of Clove

Clove Bud, Oil

Clove Bud, Extract

Clove Bud, Oleoresin

Clove Leaf Oil

Clove Stem Oil

B. Chemical Names

(none)

C. Trade Names

(none)

D. Chemical Abstracts Services Unique Registry Number

Oil of Clove - MX 8000348

Clove - 977007796

II. Empirical Formula

Clove oil, the volatiles obtained by steam distillation of whole clove, is a mixture of substances, the major components being eugenol (90%), eugenol acetate, caryophyllene and caryophyllene epoxide, (62a)

Eugenol: (4-allyl 2-methoxy phenol)	$C_{10}H_{12}O_2$
Eugenol acetate	$C_{12}H_{14}O_3$
Caryophyllene:	$C_{15}H_{24}$
Caryophyllene epoxide:	$C_{15}H_{24}O$

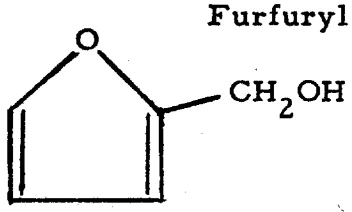
Among the minor components of the oil are the following species: (62a)

Methyl Salicylate:	$C_8H_8O_3$
Methyl-n-amy Ketone	$C_7H_{14}O$
Methyl-n-heptyl Ketone:	$C_9H_{18}O$
Furfuryl Alcohol:	$C_5H_6O_2$
α-Methyl Furfural:	$C_6H_6O_2$

Recent work has identified coniferyl aldehyde as a constituent of eugenol containing oils which include clove, pimenta and sassafras (185).

III Structural Formula

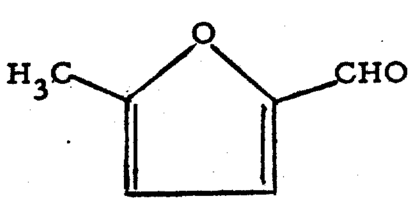
Furfuryl Alcohol



Methyl-n-heptyl Ketone

$$CH_3-\overset{\overset{O}{\|}}{C}-C_7H_{15}$$

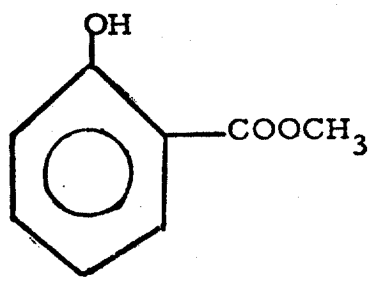
α -Methyl Furfural



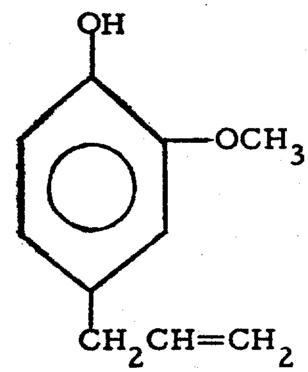
Methyl-n-amyl Ketone

$$CH_3-\overset{\overset{O}{\|}}{C}-C_5H_{11}$$

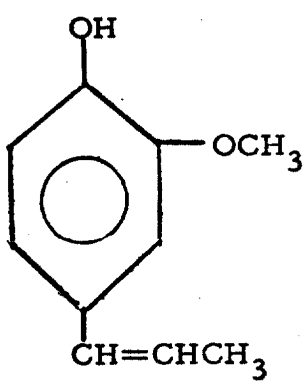
Methyl Salicylate



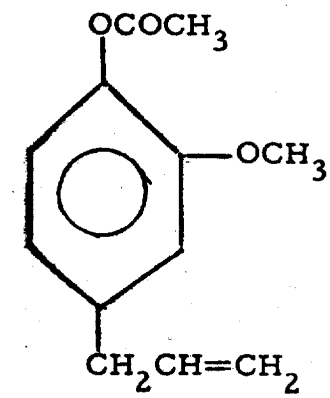
Eugenol



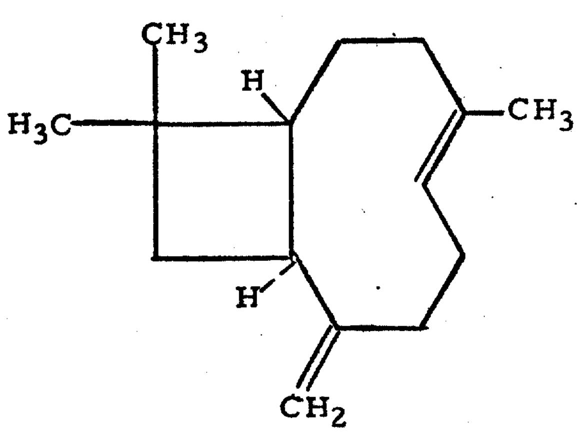
iso-Eugenol



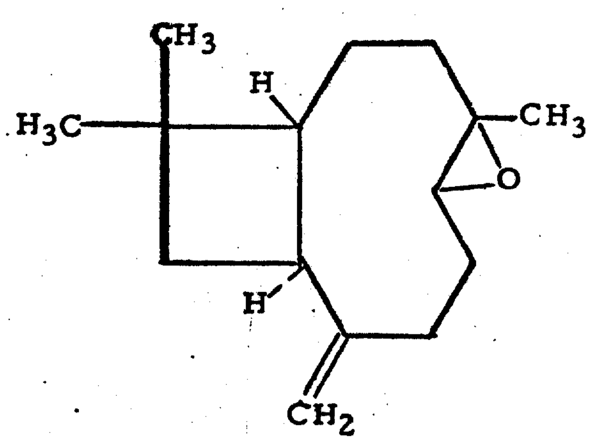
Eugenol Acetate



Caryophyllene



Caryophyllene Epoxide



IV Molecular Weight

Methyl Salicylate	152.14	Eugenol	164.20
Methyl-n-amy Ketone ...	114.20	Eugenol Acetate ..	206.24
Methyl-n-heptyl Ketone ..	142.24	Caryophyllene	204.36
Furfuryl Alcohol	98.10	Caryophyllene Epoxide	220.36
α- Methyl Furfural	110.11		

V Specifications

A - Chemical

(none)

B - Food Grade (48a)

Clove leaf oil:

Assay. Not less than 84% and not more than 88%, by volume, of phenols as eugenol ($C_{10}H_{12}O_2$).

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003%).

Heavy metals (as Pb). Not more than 40 parts per million (0.004%).

Lead. Not more than 10 parts per million (0.001%).

Clove oil

Assay. Not less than 85%, by volume, of phenols.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003%).

Heavy metals (as Pb). Not more than 40 parts per million (0.004%).

Lead. Not more than 10 parts per million (0.001%).

Phenol. Passes test.

Clove Stem oil

Assay. Not less than 89% and not more than 95%, by volume, of phenols as eugenol ($C_{10}H_{12}O_2$).

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003%).

Heavy Metals (as Pb). Not more than 40 parts per million (0.004%).

Lead. Not more than 10 parts per million (0.001%).

C. Official Compendia

Food chemicals Codex

The United States Pharmacopeia

VI Description

A - General Characteristics (48a)

Clove leaf oil:

The volatile oil obtained by steam distillation of the leaves of Eugenia caryophyllata Thunberg (Eugenia aromatica (L.) Baill.). (Fam. Myrtaceae). It is a pale yellow liquid. It is soluble in propylene glycol, in most fixed oils, with slight opalescence, and relatively insoluble in glycerin and in mineral oil.

Clove oil:

The volatile oil obtained by steam distillation from the dried flower-buds of Eugenia caryophyllata Thunberg (Eugenia aromatica (L.) Baill.). (Fam. Myrtaceae). It is a yellow to light brown liquid with a characteristic clove odor and taste. It darkens and thickens upon aging or exposure to air.

Clove stem oil:

The volatile oil obtained by steam distillation from the dried stems of the buds of Eugenia caryophyllata Thunberg (Eugenia aromatica (L.) Baill.). (Fam. Myrtaceae). It is a yellow to light brown liquid with a characteristic odor and taste. It is soluble in fixed oils, and in propylene glycol, but it is relatively insoluble in glycerin and in mineral oil.

B. Physical Properties (39a)

Clove leaf oil:

Specific Gravity @ 25°/25°C: 1.036 to 1.046.

(Correction factor from n°/n°: .00067 per°C.)

Optical Rotation: 0° to -2°.

Refractive Index @ 20°C: 1.5310 to 1.5350.

Solubility in Alcohol: Soluble in 2 volumes of 70% alcohol; a slight opalescence often occurs when additional solvent is added.

Solubility:

Benzyl Benzoate:	Soluble in all proportions
Diethyl Phthalate:	Soluble in all proportions
Fixed Oils:	Soluble often with slight opalescence in most fixed oils.
Glycerine:	Relatively insoluble
Mineral Oil:	Relatively insoluble.
Propylene Glycol:	Soluble in all proportions

Stability:

Alkali:	Unstable.
Acids:	Fairly stable in presence of mostly dilute acids.

Clove oil:

Angular rotation. Between -- 1°30' and 0°.

Refractive index. Between 1.5270 and 1.5350 at 20°.

Specific gravity. Between 1.038 and 1.060.

Solubility in alcohol at 20°. Soluble sometimes with slight turbidity, in 1 to 2 vol. and more of 70% alcohol. Only freshly distilled oils are soluble in 2.5 to 3 vol. of 60% alcohol; the addition of more alcohol causes cloudiness, except in the case of oils with a very high percentage of eugenol.

Clove stem oil:

Specific Gravity at 25°/ 25°C: 1.048 to 1.056.
(Temperature correction factor from n°/n°C =0.0006 per° C.).

Optical Rotation: $\pm$ 0° to -1° 30'.

Refractive Index at 20 C: 1.5340 to 1.5380.

Total Phenols Content: 89% to 95%.

Solubility in Alcohol: Soluble in two and more volumes of 70% alcohol.

Solubility:

Benzyl Benzoate: Soluble in all proportions.

Diethyl Phthalate: Soluble in all proportions.

Fixed Oils: Soluble in all proportions in most fixed oils.

Glycerine: Relatively insoluble.

Mineral Oil: Relatively insoluble.

Propylene Glycol: Soluble in all proportions.

Stability:

Acids: Fairly stable in the presence of weak organic acids.

Alkali: Unstable.

C. Stability in Containers, Animal feeds, etc. (48a)

Clove leaf oil:

Discolors rapidly to a brown or even purple shade when in contact with iron containers. Therefore it should be shipped preferably in glass, tin lined, stainless steel, or aluminum containers. When galvanized containers are used a precipitate may result. Store in full, tight containers in a cool place, protected from light.

Clove oil:

It darkens and thickens upon aging or exposure to air. Store in full, tight, light-resistant containers and avoid exposure to excessive heat.

Clove stem oil:

In contact with iron the oil acquires a purplish dark brown shade. Should be shipped preferably in glass, aluminum or tin-lined containers. A precipitate may result if galvanized iron containers are used. Store in tight full containers in a cool place protected from light.

VII Analytical Methods

- Identification of eugenol in mixtures by difference spectrophotometry. (35)
- Colormetric estimation of eugenol in clove oil (and other essential oils) by measuring the intensity of the blue color produced by reaction of eugenol with 2, 6-dichloroquinone chloroimide in iso-propyl alcohol. (83)
- Non-aqueous titimetry for estimation of total percent phenol compounds in clove oil. (12)
- Chromatographic resolution and identification of components in clove oil using a fractional elution proceedure applied to AL_2O_3 columns. (132)
- Specific gravity method for estimation of percent clove oil in alcoholic extracts. (132a)

VIII Occurrence and levels found in:

A. Plants

Clove is the dried flower-bud of Eugenia caryophyllata Thunberg (Fam. Myrtaceae). Clove yields, from each 100 grams, not less than 16 ml of clove oil. During 1952 a total of 1,867,560 pounds of unground cloves, and 456,816 pounds of clove oil was imported into the U. S. A. from British East Africa, Ceylone and Madagascar. (62a)

B. Animals

(none)

C. Synthetics

(none)

D. Natural inorganic sources

(none)

OIL OF CLOVE

Biological Data

I Acute Toxicity

Animal	Route	Material	LD_{50} (mg/kg)	Ref.
Rat (albino)	oral *	eugenol	1930	(166)
Rat (Osborne-Mendel)	oral **	eugenol	2680 (2420-2970)	(64, 177)
Rat (Osborne-Mendel)	oral **	iso-eugenol	1560 (1290-1880)	(177)
Mouse (Swiss)	oral **	eugenol	3000 (2400-3750)	(64)
Mouse	i. p.	eugenol	500	(20)
Mouse	i. p.	iso-eugenol (cis)	365	(20)
Mouse	i. p.	iso-eugenol (trans)	540	(20)
Guinea Pig	oral **	eugenol	2130 (1860-2430)	(64)
Dog	oral	eugenol	500	(103)

* Fasted 24 hours prior to ingestion of eugenol

** Fasted 18 hours prior to ingestion of eugenol

The pathological changes noted in the rat include the following:

> "Paralysis which occurred initially in the hind legs and the lower jaw. The forelimbs were unaffected unless general prostration or coma ensued. In those animals in which the acute symptoms subsided, the animals remained lethargic, showed signs of urinary incontinence and frequently hematuria, and gave evidence of impaired function of the hind legs for several days. Gross and microscopic observations of the tissues suggested that profound changes in fluid distribution had occurred in response to acute irritation of the gastrointestinal tract. The net impression of the effect of the eugenol, from the microscopic evidence, was essentially that of circulatory collapse with resultant congestion. (166)"

II. Short Term Studies

Daily oral doses of eugenol at 1/3 the LD_{50} values (approximately 1000 mg/kg) given to rats over a four day period produced mild liver lesions. These were characterized by slight discoloration, mottling and blunting of the lobe edges. Under the same experimental regime, no hepatotoxicity was produced by iso-eugenol. (177) In a parallel study extending over a thirty-four day period, daily doses of increasing amounts of eugenol (initially 1400 mg/kg) were administered orally to male rats. Fifteen of the animals lived long enough to receive the maximal 4000 mg/kg dose and eight survived the full thirty four days of treatment. Pathological findings were as follows; "Stomach: macrosopic--mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone--small degree of osteoporosis." (63)

In dogs where the oral LD_{50} is approximately 500mg/kg, repeated oral administration at the 200 gm/kg level (10 doses over a three week period) had no apparent change in the animals' activity and behavior, suggesting that there was no cumulative effect on the physiology at this rate and level of eugenol intake. (103)

III. Long Term Studies

In a subacute and chronic toxicity study of various food flavorings and their major components, eugenol was fed at 1000 and 10,000 ppm for nineteen weeks to male and female weanlings rats. Other groups of rats were fed iso-eugenol at 10,000 ppm for sixteen weeks. No toxic effects were noted in these animals during the period of the experiment. (63)

IV Special Studies

A number of different substances including clove oil and eugenol were evaluated as possible in vivo mucosal irritants by topical application to the pouch mucosa of Heidenhain-pouch dogs. The initial aim of the investigation was to obtain a sensitive carcinogen-receptive site for the study of carcinogenesis. It was found that clove oil, and more effectively eugenol, stimulated the production of an alkaline (~pH 8) mucous (71) while acting as powerful desquamatory agents. (167, 169) "Successive applications of the eugenol emulsion effected a progressive removal of the columnar cells from the surface of the mucosa and then from the crypts themselves, until the connective tissue matrix containing the collecting tubules of the glands was exposed. The regenerative process can be divided into three stages: (a) a preliminary resurfacing of denuded mucosa with flat and fusiform-shaped cells, which is already evident 30-60 minutes after removal of eugenol from the pouch; (b) the transformation of these new cells into low and tall columnar cells; and (c) the reformation of crypts in these areas of smoothly resurfaced mucosa. This entire process was found to occur within thirty six hours following the removal of the mucosa as far down as the bottoms of the foveolae (72)"

OIL OF CLOVE

Biochemical Aspects

The distribution and excretion routes of eugenol fed to rabbits and rats was determined using a diazo colorimetric procedure. Depending on the dose administered, within twenty four hours after peroral intake, 44% to 90% of the eugenol was found to have been absorbed, with the principal excretion route through the urine. Eugenol was detected in the blood, lungs, kidneys and liver for up to several hours after ingestion. (159)

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7

THE EFFECT OF EUGENOL AND OIL OF CLOVES ON THE GROWTH OF MICROORGANISMS

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SPICES and their essential oils have been used since antiquity. Incorporated in foodstuffs, they were the means of inhibiting bacterial fermentations and thus preventing food spoilage. McCulloch[1] records that the ancient Egyptians employed spices or their oils in embalming. Molnar[2] claims cloves was first used about the middle of the sixteenth century to alleviate toothache. Prior to this time, it had been employed to "sweeten the breath." It also was given as a remedy for gastric disorders and even placed in wounds to aid healing. Such usage, however, was strictly empirical. Scientific investigations of these substances could not be made until sufficient knowledge of bacteria and the methods of experimentation with them had accumulated. Thus, it was not until 1887 that Chamberland evaluated the antibacterial properties of a number of essential oils. This author, according to Topley and Wilson,[3] exposed anthrax bacteria and their spores to these oils in both gaseous and liquid states. He found that cinnamon, majorum, sandalwood, clove, and juniper were the most effective of the essential oils. Topley and Wilson further report that Cadeac and Meunier, in 1889, determined the antiseptic potency of various essential oils against *E. typhi* and *Pf. mallei*. It was found that whereas the control mercuric chloride, 1:1,000, destroyed these microorganisms in ten minutes, oil of cinnamon required twelve minutes and oil of cloves twenty-five minutes. Other essential oils destroyed these microorganisms only after exposure for several days, and in some instances were even ineffective after contact for ten days. The relative antibacterial potency of various essential oils was estimated by Peck,[4] 1898, as follows: cassia, 1:2,100, ceylon cinnamon, 1:2,133, cloves, 1:1,150, bay, 1:1,028, peppermint, 1:875, eucalyptol, 1:120, and eugenol, no effect. This latter finding, as will be shown later, is erroneous. MaWhinney,[5] in 1900, obtained inconsistent results in his examination of the inhibitive qualities of the essential oils and concluded that as germicides these oils were greatly overrated. Whether contamination played a role in his conflicting results should be given consideration.

The preservation of foodstuffs by means of spices or their oils was studied by Hoffmann and Evans,[6] 1911, who observed that cinnamon, cloves, and mustard were all effective as inhibitors of bacterial growth. Eugenol, 1 part per 1,000 applesauce, prevented spoilage during a fourteen-month period of storage. Bachmann,[7] 1916, also studied the preservative qualities of spices and found cloves, allspice, and cinnamon effective in preventing growth of molds and bac-

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teria. This investigator futher noted that bacteria in general were less sensitive than molds to these spices and that individual species of both groups of microorganisms varied in their sensitivity. Thus, he found that *B. subtilis* did not grow upon an agar medium which contained oil of cloves in a dilution of 1:100, while *S. macrescens* was only slightly sensitive to its presence. Fabian, Kehl, and Little,[8] 1939, investigated the role of spices in pickled food spoilage and observed that ground cinnamon and cloves were the only spices that exhibited antibacterial action in low dilution. Oil of cloves was found to restrain the growth of *B. subtilis* in a dilution of 1:100 and the *Staphylococcus aureus* in 1:800. Castell,[9] 1944, also noted that in low dilutions some of the spices, especially cloves and cassia, had inhibitory qualities. He doubted, however, if such dilutions could be used in practical application. Bullock and Lightbrown,[10] 1942, advised the addition of clove oil or cinnamon oil to infusions of quassia to prevent spoilage due to bacterial contamination.

The effect of essential oils upon pathogenic yeasts and molds has also been investigated. Myers and Thienes,[11, 12] 1925, isolated from patients suffering with a dermatitis, a yeastlike microorganism. In comparison to 1 per cent phenol, which required sixty minutes to destroy this microorganism, they found that thymol killed in one minute, cinnamon oil in thirty minutes, and clove oil in ninety minutes. Kingery and Adkisson,[13] 1928, tested a variety of pathogenic fungi against various essential oils and found the most effective in fungicidal activity were thymol, oil of cinnamon, and oil of cloves.

Oil of cloves was found to possess a phenol coefficient of 8.0 by Rideal, Rideal and Sciver,[14] 1928. Cinnamon, cassia, senfol, lemon grass, and cloves were found by Collier and Nitta,[15] 1930, to be the most effective of 106 essential oils tested. Wide variation in the dilution of the oil which inhibited growth was noted with different bacterial species. Clove oil restrained the growth of a hemolytic streptococcus in a dilution of 1:1,600, a hemolytic staphylococcus in 1:800, a mixture of five gonococcal strains in 1:1,200, *B. coli* in 1:800, and the Vibrio Nasik in 1:600. More recently, Hettche and Rosenthal,[16] 1936, tested twenty-four essential oils and observed that the oils of sandalwood, hop, cloves, and cassia possessed the most potent antibacterial properties.

A review of the literature pertaining to the antiseptic, anesthetic, and therapeutic properties of some of the essential oils was made by Petrie,[17] 1938. This author tried various combinations of essential oils as sterilizing and palliative agents in tooth cavities. For shallow cavities he advocated mixtures of oils which vaporize rapidly, such as peppermint, lemon, eucalyptus, wintergreen, and aniseed in 90 per cent alcohol. Deep cavities, he claimed, should first be painted with oil of citronella and then a pledget of cotton saturated with an alcoholic solution of oils of lemon, thymol, cloves, and cassia should be sealed in the cavity.

Recently, Shaw, Sprawson, and May,[18] 1945, stated that zinc oxide with eugenol destroyed penicillin completely. Bartels,[19] 1946, found that low-unit dilutions lost most or all of their inhibitory activity toward *Staphylococcus aureus* when mixed with eugenol zinc oxide cement, but that high-unit concentrations, though affected, still possessed definite growth-restraining action.

This review of the literature indicates that investigators in the fields of bacteriology, mycology, medicine, dentistry, and food preservation have found certain essential oils to possess antibacterial properties. Oil of cloves and its component, eugenol, is one of the more active of these oils.

EXPERIMENTAL

The following methods were employed in testing the antibacterial qualities of oil of cloves and eugenol:

1. Agar cup plate. Melted infusion agar was cooled to 45° C. and seeded with *Staphylococcus aureus* or *B. coli.* Plates were poured and when the agar was solid, small holes were bored in the center of each plate with either a lipless test tube or a cork borer. Oil of cloves or eugenol was placed in these agar depressions. A clear zone surrounding the test substance was indicative of restraint of microbic growth.

2. Infusion agar, 100 ml. amounts, was adjusted to pH 6.0, pH 7.0, and pH 8.0, respectively. Oil of cloves or eugenol was added to each lot to give a concentration of 0.5 per cent. Plates were poured and after solidification, one-half of the medium was removed and replaced with plain infusion agar of the same pH reaction. The surface of the medium was inoculated in parallel streaks with *B. subtilis, Staphylococcus aureus, B. coli* and *B. pyocyaneus.* Each streak of inoculum was begun on the plain infusion agar portion and carried over onto the agar containing the clove oil or eugenol. Antibacterial properties were indicated by the restraint of growth on the clove agar and the plain infusion agar neighboring it. (See Table I and Figs. 1 and 2.)

TABLE I. THE EFFECT OF CLOVE OIL AND EUGENOL IN AGAR MEDIUM OF DIFFERENT pH REACTIONS

REACTION	MICROORGANISM	GROWTH INHIBITION IN MM. ZONE ADJACENT TO* EUGENOL AGAR	CLOVES 1 AGAR†	CLOVES 2 AGAR†
pH 6.0	*B. subtilis*	++++	++++	++++
pH 7.0	*B. subtilis*	++++	+++	+++
pH 8.0	*B. subtilis*	++++	+++	+++
pH 6.0	*Staphylococcus aureus*	+++	++	++
pH 7.0	*Staphylococcus aureus*	+++	++	+++
pH 8.0	*Staphylococcus aureus*	+++	++	++
pH 6.0	*B. coli*	+++	++	++
pH 7.0	*B. coli*	++	++	++
pH 8.0	*B. coli*	++	++	++
pH 6.0	*B. pyocyaneus*	Unaffected	Unaffected	Unaffected
pH 7.0	*B. pyocyaneus*	Unaffected	Unaffected	Unaffected
pH 8.0	*B. pyocyaneus*	Unaffected	Unaffected	Unaffected

*Inhibition of 16 to 20 mm. designated by ++++; 11 to 15 mm. by +++; 6 to 10 mm. by ++. *B. pyocyaneus* grew well even upon the portion of medium containing either the eugenol or oil of cloves.

†Two manufacturers' brands of cloves.

3. Agar in test tubes, cooled to 45° C., was seeded with various microorganisms and poured in plates. Small amounts of eugenol alone or mixed with zinc oxide or zinc oxide alone were placed on the surface of the medium. Zones of growth inhibition about the test materials indicated sensitivity of the microorganisms. (See Table II.)

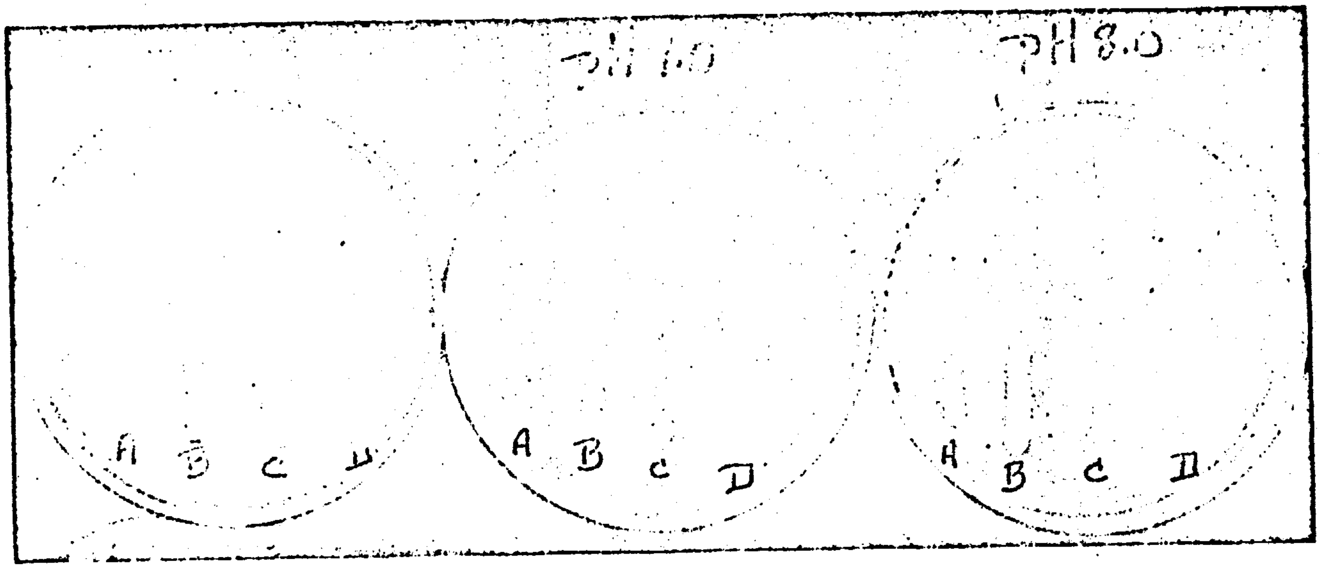

Fig. 1.—Forty-eight hours' incubation. Upper half plate (test portion), infusion agar plus 0.5 per cent eugenol. Lower half plate (control portion), plain infusion agar. *A, B. subtilis; B, Staphylococcus aureus; C, B. coli; D, B. pyocyaneus.* Note, *B. pyocyaneus* grows on the test portion. Note also the absence of growth along heavy inoculated streak lines of other microorganisms on test portion and adjacent control portion. (See results, Table I.)

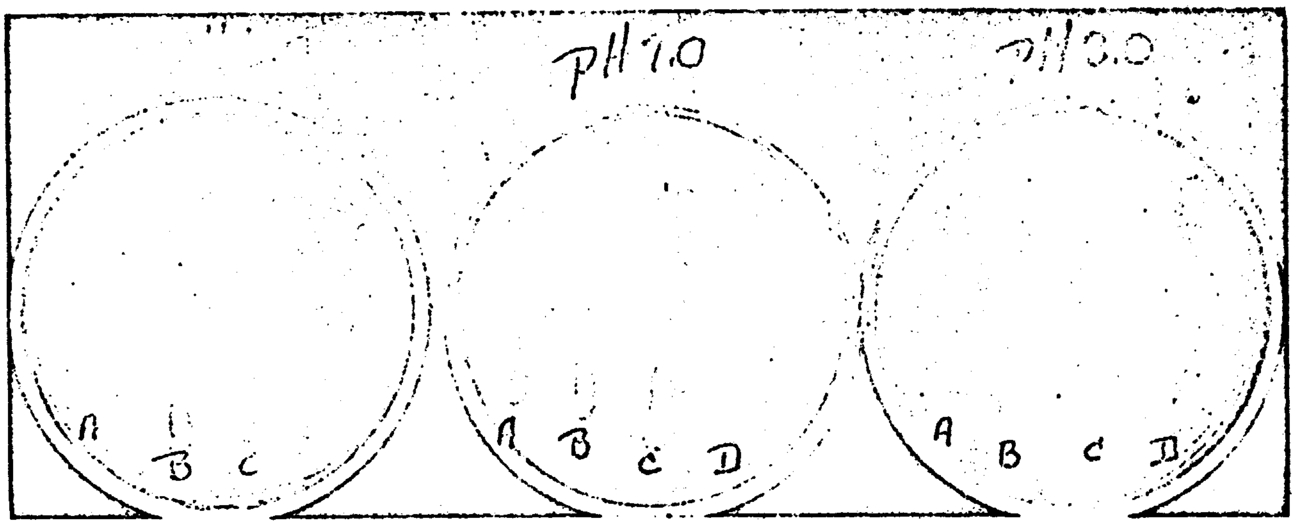

Fig. 2.—Test portion contains oil of cloves, 0.5 per cent. Otherwise same as Fig. 1.

TABLE II. EFFECT ON GROWTH OF MICROORGANISMS OF EUGENOL, EUGENOL-ZINC OXIDE, AND ZINC OXIDE

MICROORGANISM	GROWTH INHIBITION IN MILLIMETERS*		
	EUGENOL	EUGENOL-ZINC OXIDE	ZINC OXIDE
Staphylococcus aureus	6	6	0
Staphylococcus albus	5	4	0
Staphylococcus citreus	5	5	0
Serratia marcescens			
(Col. strain)	6	5	0
(Feb. strain)	6	5	0
Monilia albicans	9	7	0
B. subtilis	5	5	0
E. typhi	9	7	0
E. coli	5	5	0
B. Friedländer	4	3	0
B. proteus	5	4	0
B. pyocyaneus			
(Col. strain)	1	1	0
(New York University strain)	1	1	0
(New York State Laboratory strain)	0	0	0

*Area measured was from the periphery of test material to region of colony development.

4. Infusion agar plates were inoculated in parallel streaks with four test microorganisms mentioned previously. In the center of each streak was placed either zinc oxide or zinc oxide with eugenol. Interruption of growth along the streak lines in the vicinity of the test material indicated inhibitory activity. (See Fig. 3.)

5. Eugenol, 10 per cent, in aqueous suspension, alcoholic solution, or detergent (oxydon) suspension was added in variable amounts to 10 ml. volumes of infusion broth, which previously was inoculated either with *Staphylococcus aureus* or *B. pyocyaneus*.

RESULTS

The following information was obtained from the preceding experiments:

1. Microorganisms vary in their sensitivity toward eugenol or oil of cloves. *E. typhi* and *Monilia albicans* (*Candida albicans*) were affected to a greater extent than the other microorganisms tested. *B. pyocyaneus* (*Ps. aeruginosa*) was the most resistant. Three strains of this bacterium obtained from different sources (Columbia University, New York University, and New York State Laboratory) were tested and all showed similar resistance toward oil of cloves or eugenol. Other studies are being made on this subject.

2. Variation in hydrogen-ion reaction between pH 6.0, pH 7.0, and pH 8.0 had little effect on the inhibitory qualities of either oil of cloves or eugenol.

3. Zinc oxide has no apparent antibacterial effect in the dried state on microorganisms. Even when added to infusion broth in 1 per cent amounts, it did not cause destruction of either *Staphylococcus aureus* or *B. pyocyaneus* during a three-day period of exposure.

4. Oil of cloves or eugenol when incorporated into a paste with zinc oxide still possesses definite inhibitive properties.

5. Eugenol in alcoholic solution was more effective than when suspended in water or detergent. This no doubt is due to the fact that the aqueous and de-

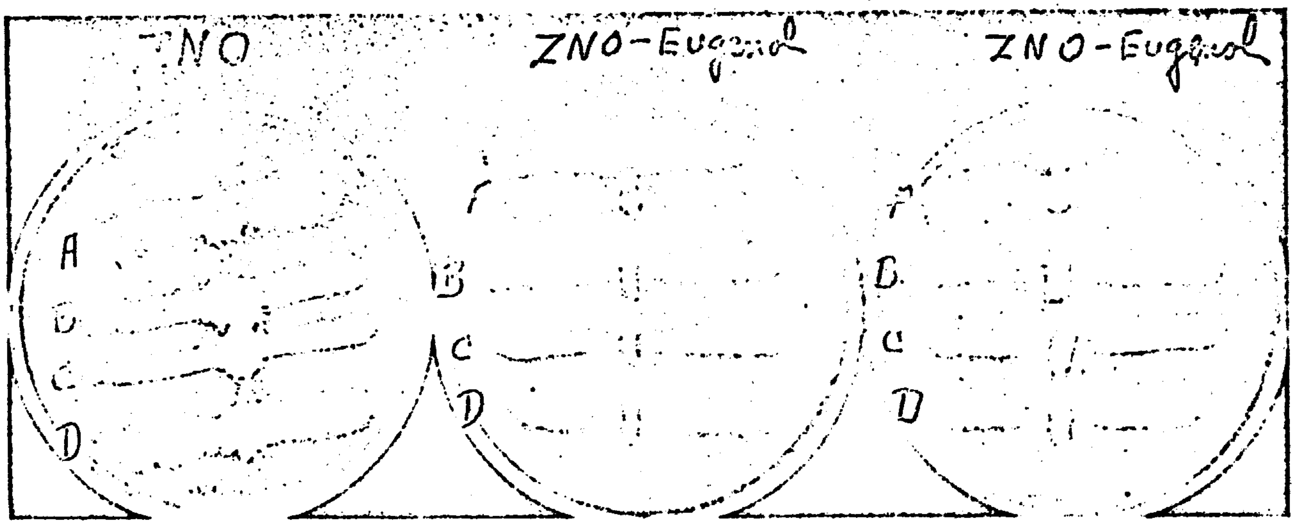

Fig. 3.—Forty-eight hours' incubation infusion agar. *A, B. pyocyaneus; B, B. coli; C, Staphylococcus aureus; D, B. coli.* Note that zinc oxide does not restrain growth, whereas zinc oxide-eugenol paste, large or small amount, inhibits growth of microorganisms with exception of *B. pyocyaneus.*

tergent suspensions either separate out in the form of globules or sediment at the bottom of the tubes of broth, and so act only in a restricted area of the broth, allowing growth to occur in the upper part of the tube.

6. Oil of cloves and eugenol were comparable in their antibacterial properties. Peck's observation that eugenol was without effect was due possibly to use of an inferior brand of eugenol or to the presence of a resistant microorganism in specimens of saliva which he employed as inoculum. Our results with *B. pyocyaneus* indicate this latter possibility.

SUMMARY

Bacterial species vary in their individual sensitivity to either oil of cloves or eugenol. *B. pyocyaneus* (*Ps. aeruginosa*) was markedly resistant. Variation in hydrogen-ion concentration had no appreciable effect on the activity of clove oil or eugenol. Incorporated in a paste with zinc oxide, eugenol, or oil of cloves still retains definite antibacterial properties.

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Analysis of Phenol-Containing Volatile Oils

12

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▶ **Phenol-containing volatile oils are analyzed by nonaqueous titrimetry, with sodium methoxide in benzene-methanol as titrant. Dimethyl formamide, acetonitrile, and ethylenediamine are used as solvents. The titrations are performed potentiometrically with a sleeve-type calomel and platinum electrode system. A number of advantages over the conventional extraction procedure are indicated.**

The phenol content of volatile oils is usually estimated by extraction with alkali solution. Guenther (*6*) describes the method and its modifications and points out its sources of error and weaknesses. The extraction method is recognized by the official drug standards (*11, 12*) for the analysis of several phenol-containing volatile oils.

Although numerous procedures involving nonaqueous titrimetry are reported for the analysis of phenolic compounds, little has been done to apply these methods to volatile oils.

Cundiff and Markunas (*4*) report the successful potentiometric titration of thymol, a phenol found in thyme oil, using pyridine as the solvent. The visual titration proved unsuccessful. No data were given.

Warner and Haskell (*13*) successfully titrated thymol with sodium methoxide in benzene-methanol. The solvent was butylamine. They employed a specially prepared antimony electrode and a glass electrode. A titration curve is shown but no data are given to indicate the quantitative nature of the procedure.

Butler and Czepiel (*3*) determined phenolic groups in lignin preparations. They used an antimony-saturated calomel electrode system and dimethyl formamide as the solvent. The titrant was potassium methoxide in benzene-methanol. Eugenol and isoeugenol, common volatile oil constituents, were studied as model compounds. Suitable titration curves were obtained.

Guenther and Langenau (*9*) in a review article on essential oils recognize the potential application of nonaqueous titrations to the analysis of volatile oils containing phenolic constituents.

This paper reports the successful titration of certain phenols as pure compounds and as constituents in volatile oils. A platinum and calomel electrode system was employed. The solvents were dimethyl formamide, acetonitrile, and ethylenediamine. The procedure has merit as a general method for analyzing phenol-containing volatile oils.

EXPERIMENTAL

Apparatus. The phenols and volatile oils studied were titrated potentiometrically with a Fisher Titrimeter equipped with a sleeve-type calomel electrode and a platinum electrode. A 150-ml. beaker served as the titration cell and was covered with a piece of heavy cardboard to protect the contents from carbon dioxide and moisture in the air during the titration. Three holes in the cardboard cover admitted the electrodes and buret tip into the titration beaker. A magnetic stirrer and a glass-covered stirring bar were used to stir the solution during titration. The titrant was stored in the reservoir of an automatic buret and in titration was delivered from a 50-ml. buret. Titrations were performed in a specially constructed hood to protect the operator from the caustic fumes of the chemicals.

Reagents and Chemicals. Sodium, reagent grade, Merck.

Sodium methoxide, 0.1*N*, prepared as described below.

Absolute methanol, reag... Merck.

Benzene, anhydrous, reag... Merck.

Benzoic acid, primary sta... Merck.

Ethylenediamine, 95 to 10... man.

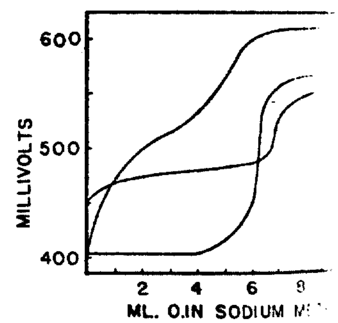

Figure 1. Titration of th... thyme oil

A. In dimethyl formam...
B. In acetonitrile
C. In ethylenediamine

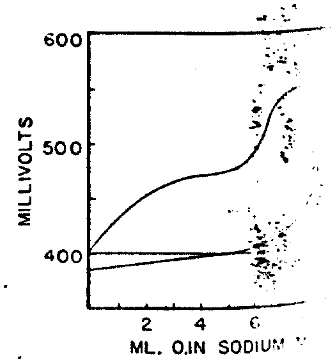

Figure 2. Titration of ... oil, and bay oil

A. In dimethyl fo...
B. In acetonitri...
C. In ethylene...

Table I. Analysis of Phenols and Phenol-Containing Volatile Oils

Phenol or Volatile Oil	Variety	Requirement	Solvent	% Phenols	Chief Constituent
Thymol	N.F. X		Dimethyl formamide	98.37 ± 0.16 (5)[b]	Thymol
Thyme oil (red)	N.F. VII	n.l.t.[a] 20% phenols	Dimethyl formamide	28.89 ± 0.43 (5)	Thymol
			Acetonitrile	28.67 ± 0.38 (4)	Thymol
			Ethylenediamine	28.80 ± 0.08 (4)	Thymol
Eugenol	U.S.P. XV		Dimethyl formamide	97.70 ± 0.19 (3)	Eugenol
			Acetonitrile	100.15 ± 0.15 (3)	Eugenol
Clove oil	U.S.P. XV	n.l.t. 85% phenols	Dimethyl formamide	88.82 ± 0.13 (4)	Eugenol
			Acetonitrile	89.01 ± 0.41 (3)	Eugenol
			Ethylenediamine	89.71 ± 0.15 (3)	Eugenol
Wintergreen oil	U.S.P. XV	n.l.t. 98% methyl salicylate	Dimethyl formamide	99.38 ± 0.24 (5)	Methyl salicylate
			Ethylenediamine	99.18 ± 0.18 (4)	Methyl salicylate
Carvacrol	Commercial		Dimethyl formamide	100.05 ± 0.35 (4)	Carvacrol
			Acetonitrile	100.57 ± 0.23 (4)	Carvacrol
			Ethylenediamine	99.57 ± 0.43 (4)	Carvacrol
Origanum oil	Commercial	63–74% phenols	Dimethyl formamide	74.57 ± 0.07 (3)	Carvacrol
			Acetonitrile	74.44 ± 0.40 (3)	Carvacrol
			Ethylenediamine	75.14 ± 0.06 (3)	Carvacrol
Bay oil	N.F. X	50–65% phenols	Dimethyl formamide	63.86 ± 0.35 (5)	Eugenol
			Acetonitrile	64.24 ± 0.46 (4)	Eugenol
			Ethylenediamine	63.11 ± 0.22 (4)	Eugenol
Isoeugenol	Commercial		Dimethyl formamide	100.01 ± 0.28 (4)	Isoeugenol
			Acetonitrile	99.67 ± 0.42 (4)	Isoeugenol
			Ethylenediamine	99.38 ± 0.24 (4)	Isoeugenol

[a] Not less than.
[b] Number of determinations.

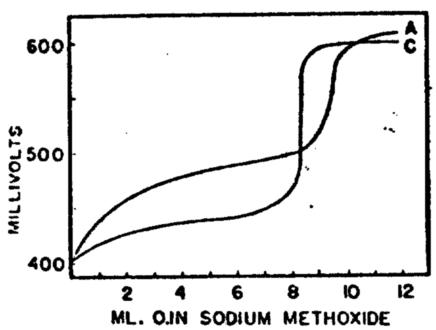

Figure 3. Titration of methyl salicylate

A. In dimethyl formamide
C. In ethylenediamine

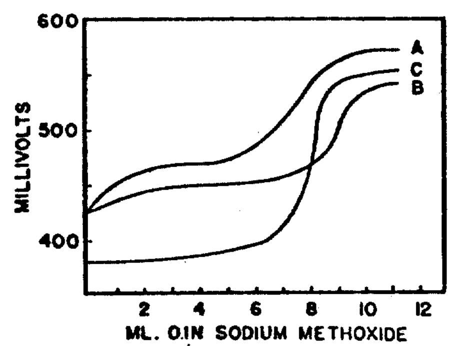

Figure 4. Titration of carvacrol and origanum oil

A. In dimethyl formamide
B. In acetonitrile
C. In ethylenediamine

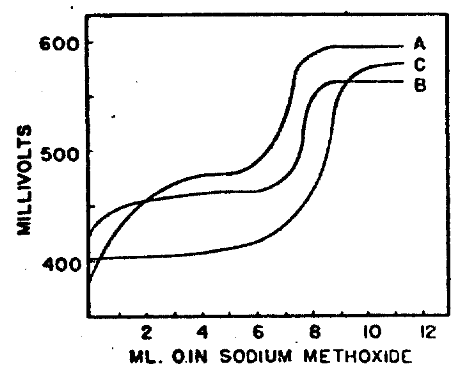

Figure 5. Titration of isoeugenol

A. In dimethyl formamide
B. In acetonitrile
C. In ethylenediamine

Dimethyl formamide, technical, Du Pont.

Acetonitrile, reagent grade, Fisher.

Azo violet (p-nitrobenzeneazoresorcinol) indicator solution, saturated solution in benzene.

Phenols and volatile oils, varieties indicated in Table I.

Sodium Methoxide Solution, 0.1N. Approximately 5 grams of clean sodium was added slowly to 100 ml. of absolute methanol in a flask immersed in an ice bath. After all the sodium dissolved, 150 ml. more of methanol was added, followed by 1500 ml. of dry benzene. The solution was stored in the reservoir of an automatic buret protected from moisture and carbon dioxide in the atmosphere. Prior to the titration of a sample, 50 ml. of titrant was transferred from the automatic buret to the buret of the titration assembly.

The sodium methoxide solution was standardized against benzoic acid (*1*).

Procedure. Approximately 30 ml. of solvent was placed in a 150-ml. beaker. A stirring bar was added and the solution was magnetically stirred. Three drops of azo violet indicator solution were added and the solvent was titrated visually with 0.1N sodium methoxide solution to the first permanent clear blue color. Less than 0.05 ml. of titrant was usually required to neutralize the acidic impurities of the solvent. One to 2 meq. of the phenol was accurately weighed and added to the titration beaker. For the volatile oils an amount was weighed which contained approximately 1 to 2 meq. of the phenolic constituent. This was most conveniently done by placing the oil in a 2-dram dropper bottle. The system was weighed, oil was transferred by dropper to the titration beaker, and the system was again weighed. The solution, magnetically stirred, was titrated with 0.1N sodium methoxide solution, using the Fisher Titrimeter equipped with a sleeve-type calomel and platinum electrode system. Increments of 0.1 ml. of titrant were added in the vicinity of the end point. The end point was indicated by the inflection in the curve obtained by plotting millivolts against titrant added. The exact end point was determined by plotting $\Delta E/\Delta V$ vs. V (ml.).

DISCUSSION

The standard method for analyzing volatile oils which contain phenolic constituents is by extracting the oil with aqueous alkali solution and measuring the amount of extracted material in a cassia flask. According to Guenther (*6*), the method was first used by Gildemeister for the estimation of phenols in thyme oil. Guenther discusses the procedure, its modifications, difficulties, and shortcomings. The disadvantages have been indicated in a paper (*2*) dealing with colorimetric determination of thymol in thyme oil.

and volatile oils investigated.

Typical titration curves in the different solvents are shown in Figures 1 to 5. The most basic solvent, ethylenediamine, produced the greatest inflections. The end point may be obtained from the curves by inspection. Phenols are too weak to titrate in water, but they behave as weak acids in ethylenediamine. Although the inflections are not so marked with dimethyl formamide or acetonitrile as the solvent, the end point is readily obtained from a differential plot or even by inspection—for example, isoeugenol.

Titration curves for thymol and thyme oil, the chief phenolic constituent of which is thymol, are shown in Figure 1. Thyme oil (red variety), N.F. VII (*10*) (containing not less than 20% phenols), was used in this investigation. According to N.F. X (*11*) thyme oil must contain not less than 40% phenols to meet official requirements. The results using the three solvents are in excellent agreement. The determination of thyme oil in dimethyl formamide by the procedure described was reported in an earlier paper (*2*). A colorimetric method, the conventional procedure, and the titrimetric analysis yielded comparable results with the red and white varieties of thyme oil.

Clove oil, bay oil, and eugenol (the main constituent in clove and bay oils) show similar titration curves (Figure 2) in the three solvents. The curves for eugenol and isoeugenol (Figure 5) compare favorably with those shown by Butler and Czepiel (*3*). The results in Table I for clove and bay oils and the nature of the curves demonstrate the applicability of nonaqueous titrations to the analysis of volatile oils of this type.

65% phenols. According to Palkin and Wells, as reported by Guenther (*8*), 89.3% of the phenol content is eugenol, while the remaining 10.7% is chavicol (*p*-allylphenol). The calculation of phenol content in bay oil shown in Table I is based on eugenol.

Methyl salicylate is the principal constituent of several volatile oils (wintergreen oil, sweet birch oil) or it may be prepared synthetically. Phenolic esters of this type were successfully titrated as weak acids in ethylenediamine by Glenn and Peake (*5*). Typical titration curves in dimethyl formamide and ethylenediamine are shown in Figure 3.

Titration curves for origanum oil and carvacrol are shown in Figure 4. Origanum oil contains 63 to 74% phenols (7) consisting mainly of carvacrol. Isoeugenol occurs in varying concentrations in a number of volatile oils. Typical titration curves in the three solvents are shown in Figure 5.

The titrimetric procedure offers a number of advantages over the classical method for analyzing volatile oils which contain phenolic constituents. Once the titrant has been prepared and standardized, routine analyses can be effected in a short time. Sample weights of less than 1 gram are needed, whereas the conventional method requires 10 ml. As volatile oils are generally rather expensive, this may be an important economic consideration. Percentage content is expressed in terms of weight in weight rather than volume in volume. The former is the usual manner for expressing the concentration of constituents. The accuracy and precision are superior to those obtainable by the extraction procedure. No problem of meniscus or solubility of non-alkali-soluble substances.

Although a Fisher Titrim... used, any suitable potentiom... be employed.

ACKNOWLEDGMENT

The author thanks Fritzsche... ers, Inc., New York 11, N. Y., for supplying the bay oil, isoeug... origanum oil used in this inves... He wishes to thank Jack A... preparing the curves.

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Automatic Unit for Determination of Volatile Matter in Coal, Coke, and Char

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Research and Development Division, Pittsburgh Consolidation Coal Co., Library, Pa.

▶ In determining the volatile content of coal, coke, and char, the American Society for Testing Materials designates a 7-minute heating period at 950° C. with modification of the heating rate for certain nonagglomerating materials. Conventional manual control prevents close duplication of heating rates and results are often erratic. The apparatus described permits close control of the heating, is sufficiently flexible to be adapted to a variety of materials, and automatically controls the entire operation. It is designed to operate with one or two vertical tube furnaces.

In determining the volatile matter of coals and cokes, the American Society for Testing Materials designates a 1-gram portion of sample weighed into a 10- or 20-ml. platinum crucible and lowered into an electrical vertical tube furnace or heated in a muffle t... After a 7-minute residence th... crucible and contents are ... cooled, and weighed (*1*). Th... content is calculated from th... loss.

The shock heating effect ... above treatment is too extre... tain types of samples and ... losses occur. These lo... fested by "sparking" of the ... ticles in the hot portion of th...

EFFECTS OF HISTAMINE AND EUGENOL ON GASTRIC MAST CELL DIAPEDESIS AND LEUCOPEDESIS IN THE ALBINO RAT [1]

PERIHAN CAMBEL, JAMES T. SCOURIS and CECILIA E. CONROY

Evidence was brought by Cambel *et al.* (1952) that mast cells were present in the stomach wall as well as in smears obtained from the gastric juice of albino rats. The authors concluded, therefore, that migration of these cells by means of their ameboid movement occurred from the stomach wall into the gastric lumen. It seemed of interest to investigate if histamine or the gastric irritant eugenol (Hollander and Lauber, 1948) would effect gastric mast cell diapedesis. Stained smears from the gastric secretion on the stomach wall were prepared as previously described (Cambel *et al.*, 1952).

All experiments were conducted on albino rats of the Sprague-Dawley-Holtzman strain. They were fed Purina Dog Chow *ad libitum*. Histamine administration: Fifteen female and 15 male rats were supplied with tap water [2] *ad libitum*. The average age of the animals was 6 weeks at the onset of the experiment, while their weights ranged from 165 to 240 g. Daily injections of 1 ml. of an aqueous histamine dihydrochloride solution in a concentration of 0.040 mg. ml. were given over a period of one, two and four weeks. Totals of 0.28 mg. of histamine were administered for one week, 0.56 mg. for 2 weeks, and 1.12 mg. for 4 weeks. Eugenol administration: Twelve female and 12 male rats, aged from 5 to 8 weeks, and weighing from 136 to 190 g. at the onset of the experiment were used. They were kept without water supply and conditioned to drinking from a pipette daily 24 ml. of a 0.1% and 6 ml. of a 1% aqueous eugenol emulsion mixed in a Waring Blendor according to Kraus and Hollander (1949). Tergitol-Penetrant [3] (1.50%) was used to stabilize the emulsion. Eugenol

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[1] A contribution from the Cancer Research Laboratory, University of Florida, Gainesville, Florida.

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[2] The water supply of Gainesville is fluorinated to a concentration of 1 part/1,000,000.

[3] Tergitol 7 was kindly supplied by Carbide and Carbon Chemical Company.

was administered for a one week period and a 4 weeks period.

The findings in the smears of the non-treated controls were reported previously (Cambel *et al.*, 1952). The smears taken from the histamine-treated rats showed microscopically a progressive decrease of the bacterial flora as the period of treatment was augmented. Usually, the numbers of polymorphonuclear leucocytes, squamous and columnar cells decreased as compared to those in non-treated rats. In a 1 week histamine-treated rat, lymphocytes and in a 4-week histamine-treated animal, a few erythrocytes were noted. In only one 2-week treated rat were mast cells present, while in all the others no mast cells or free metachromatic granules could be detected.

In the smears from the eugenol treated animals a definite decrease in the bacterial flora was also noted. The greatest decrease was observed in the 4-week treated rats. In only one animal could polymorphonuclear leucocytes be distinguished among the cellular debris. No mast cells or free metachromatic granules were seen.

Summary

Histamine dihydrochloride as well as eugenol, as administered above, impaired both gastric mast cell diapedesis and gastric leucopedesis (Tomenius, 1947) in the albino rat. Both substances depressed the bacterial flora in the gastric juice.

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ORIGINAL REPORTS

Hypothermic activity in the eugenol and safrol series

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(*) Report presented at the Academy of Pharmacy, session of March 30, 1960.

Hypothermia, a constant sign of acute intoxication by phenolic substances, ha been pointed out by BINET, since 1896, in the symptomatology of accidents triggered by intraperitoneal injection of eugenol in the Dog (4).

During recent acute toxicity studies in the eugenol, methyleugenol and safrol series we have observed the frequency of hypothermias in the Mouse. Despite the chemical homogeneity of the various bodies studied, no relationship appeared between acute toxicity and the intensity of thermic drops; this led us to a systematic search of eventual relationships between the structure and the hypothermic activity (5,6,7,8,9).

For the Mouse, via intrapertoneal route, the toxicity characteristics of the twelve bodies studied are grouped in Table I.

TECHNIQUE

We deemed it useful to define the conditions permitting the valid utilisatin of the white Mouse (Swiss variety) for the research undertaken.

In fact one cannot fail to be surprised by the diversity of the values indicated as the central temperature of the Mouse. No doubt the discretion of certain authors (Ch. RICHET, E. GIEY) of previous years is explained by the necessity of very fine thermoelectric probes to take, in all objectivity, the rectal temperature of a small mouse, but the disagreements of modern data are "a priori" less evident.

L. THER (14), in his PHarmakologische Methoden, indicates 36 - 38°C as the normal temperature of the Mouse. Various researchers, such as Z. ASCODI (1), H. G. BARBOUR and J. TRACE indicate 38.1°C as a normal temperature. According to L. P. HERRINGTON, at an ambient temperature of 26.7°C the normal temperature would be 36.5°C ± 1.3°C. ASCODI gives values generally less than 37°C; BENEDICT and his coworkers have insisted on the importance of these divergences, without supplying an explanation for them (3).

More recently W. USINGER (15) has again questioned all the data acquired. With mice placed on a well defined diet, W. USINGER has established the correlations of ambient temperature and rectal temperature: - in neutral metabolic zone, at an ambient temperature of 30°, the rectal temperature is 35.1°C $\pm$ 0.73°C; for an ambient temperature of 25°, the average normal rectal temperature is 35.9°. W. USINGER ascribed the disagreement between his results and the data acquired to the inertia of the measuring instruments used.

In some older work W. HEUBNER and W. SILBER (13) pointed out the extreme thermic instability of the Mouse, whose rectal temperature can vary by 2.2°C during the day (outside temperature 18 - 20°C); but the same Mouse kept in an enclosure at 25°, and the animals having adapted to several temperature readings without disturbing motor reactions, the rectal temperature oscillations during a ten hour interval are less than 0.6° for 75% of the subjects.

Through our own tests we quickly recognized that the simple fact of penning a new mouse and introducing the thermoprobe for the first time results in an appreciable thermic perturbation. A psychic shock takes place, triggering defense reflexes resulting in a motor agitation which the restraining of the animal eliminates only in appearance: the rectal temperature rises.

The physiologists being in disagreement concerning the temperature of the Mouse corresponding to the zone of indifferent metabolism, no precise data appearing concerning the value of the reactional thermic perturbation occurring after the introduction of the thermoprobe, we systematically determined on 2500 white mice (Swiss), kept at 25 $\pm$ 1° (NETHEIER and HINZ Thermorapid apparatus - 1.3 mm diameter probes):

1. The instantaneous thermometric data a read two and four seconds after the introduction of the thermocouple;

2. The thermometric data b read five minutes after the introduction of the thermocouple;

3. The thermometric data c read fifteen minutes after the introduction of the thermocouple.

The reading times were determined: - for the value a in function of the inertia of the measuring instrument used; - for the value b because the temperature rise generally reaches its maximum at the fifth minute; - for the value c because in general the duration of the hyperthermic perturbation equals at most eighteen minutes.

Subjected to the same diet,handled in the same conditions, the 2500 mice tested appeared healthy; all came from the same colony and samples taken at random produced normal growth curves. We must point out however that no parasitologic testing had been done, which is unquestionably a fault.

The a temperatures showed considerable variations; the value b - a is never negative, is rarely nil and most often varies between 0.5° and 0.7°, but it can substantially exceed 1°; the c value is generally very close to a, if not equal.

Can the average of the a temperatures measured be considered as the mean rectal temperature of the Mouse? To confirm this the inertia of the measuring instruments should be substantially nil, or at least less than the time necessary for the animal to affirm the reactions which perturb its central temperature. Given the thermoregulating system of the Mouse (and no doubt also that of other small rodents), it seems that the knowledge of this central temperature must remain indeterminate within the meaning of HEISENBERG. However we have observed that mice adapted to repeated measurements, i.e. trained mice, exhibit progressively decreasing thermic reactions(1) in 80% of the cases.

(1) In this connection, the study of nyctothemeral variations of the central temperature of the Mouse could be undertaken; this study is extremely delicate for the nutritional requirements of the Mouse, which are normally satisfied in an almost continuous fashion, tend to block the pure nyctothemeral rhythm since the animal cannot be kept at ideal rest. In this connection see similar work performed on the Rat by A. HEUSNER (12) and C. H. MARX (11).

Tables II and III present our experimental results (total of 2500 animals)

Table III shows that the reactional thermic perturbation is all the more intense as the animal has a lower a temperature, which is normal.

At the fifteenth minute the values of c are identical to within less than 10% to those of a in 90% of the cases and, at the eighteenth minute, in 98% of the cases.

The body of observations we present confirms the conclusions of W. HEUBNER and of W. SILBER: the Mouse can be used to test hypo- and hyperthermic substances on the absolute condition that exact account be taken of the particularities offered by its thermoregulation. All animals must be tested, isolated, at rest at a well defined temperature, always the same, comprised between 25 + 1° and 30 + 1°. The corresponding a, b and c temperatures must be recorded and the product to be studied must be administered only a minimum of fifteen minutes after the insertion of the probe. The effects obtained must be calculated not with respect to the value b but with respect to the value c.

Comparative tests call for the selection of animals for which the values of a remain closely grouped. For practical reasons we used the group for which the following relationship was satisfied;.

39.9° is less than a is less than 38.6°

The products injected were dissolved at 5% in neutral olive oil; we made certain that the intraperitoneal injection of 0.2 cc of neutral olive oil caused no perturbation in the rectal temperature of the Mouse.

The quantities of products injected were always less than the infralethal amount. For each quantity tested the tests involved 20, 30 or 40 animals.

TESTS IN THE EUGENOL SERIES

Eugenol, cis-eugenol, trans-eugenol and dihydroeugenol are all hypothermic agents with clear and rapid effects, but of variable intensity. These effects, even at low dosage, are already manifest less than one hour after the injection; their duration, at doses near the index of aggressivity, is maximal in the case of dihydroeugenol (See Table VI).

The maximal intensities of these effects can be compared at doses of equal weight (which are substantially isomolecular), or m re usefully at equivalent aggressivity, - in this last case the reference dose adopted corresponds to one half the index of aggressivity. Tables IV and V express these results.

At the limits immediately below the index of aggressivity dihydroeugenol and eugenol produce extremely spectacular drops in temperature (See Table VI).

TESTS IN THE METHYL-EUGENOL SERIES

Methyleugenol, cis-eugenol, trans-methylisoeugenol and dihydromethyleugenol are hypothermic agents; their effects are progressive, they reach their maximum values after only a fairly long period, but their duration is considerable.

Tables VII, VIII and IX make it possible to compare these effects with those determined by the homologs of the eugenol series.

The hypothermic activity of the substances of the methyleugenol series being fairly slow to establish itself fully, it was not feasible to experiment at doses near the LD_{50} limit, the times of crisis being furthermore comprised between one and three days.

In Table IX we group some results observed at doses representing approximately half the index of aggressivity.

Curves 3 and 4 take account of the evolution of hypothermia in the methyleugenol series.

TESTS IN THE SAFROL SERIES

Safrol, cis- and trans-isosafrols and dihydrosafrol are less toxic than their homologs of the eugenol and methyleugenol series, - but their times of crisis are high.

Safrol and dihydrosafrol are very little active, on the other hand the two isosafrol stereo-isomers are hypothermic agents of durable efficacy, though they take a fairly long time to manifest themselves. Table X indicates the results obtained with the unit dose of 0.10 g/kg; table XI presents the results corresponding to the administration of half the infralethal dose, which quantity is of the order of 0.10 g/kg for safrol and cis-safrol; finally table XII indicates the results obtained with a dose corresponding substantially to one fourth the index of aggressivity for safrol and for the two isosafroles;- since the too low toxicity of dihydrosafrol did not permit comparable experimentation, the maximum dose administered did not exceed 0.50 g.

Curves 5 and 6 indicate the evolutio of the hypothermia caused by the two isosafrols.

DISCUSSION

Any temperature drop less than 1° in absolute value will be considered as not significant; likewise any observation extended beyond six hours is not considered as valid since the Mouse cannot be deprived of food for more than a few hours without ceasing to be "normal".

Taking account of the forementioned reservations, one can witness:

1. The very small activity, or inactivity, of safrol and dihydrosafrol;

2. The powerful and rapid, but short lived activity of eugenol and dihydroeugenol;

3. The less immediate but more sustained activity of dihydromethyleugenol, of methyleugenol, and especially of methylisoeugenols and isosafrols. In view of their low toxicity isosafrols offer a certain practical interest as mild hypothermic agents with a durable action.

The pattern of hypothermia induced dissociates the eugenol series, which possesses a free phenol function, from the methyleugenol and safrol series, which no longer possess a free phenol function. From the structural point of view it is odd to find that the safrol series, although less toxic, offers a hypothermic activity just as pronounced as the methyleugenol series, - and even more pronounced at isotoxic doses. This example, which is far from being unique, shows the care needed in distinguishing a pharmacodynamic activity and a toxic aggressivity, even in a homogeneous series.

(Pharmacy and Pharmacodynamics Laboratories, Faculty of Medicine and Pharmacy, Toulouse).

TABLE I.

Substance	In 24 hours DMJM	LD 50 (Kaerber)	DmTM	Index of aggressivity	Infralethal dose
Eugenol	0.40	0.50	0.60	0.50	0.40
Isocugenol cis	0.10	0.365	0.60	0.34	0.10
Isocugenol trans	0.20	0.54	0.80	0.54	0.20
Dihydroeugenol	0.55	0.83	1.00	0.78	0.55
Methyleugenol	0.50	0.97	1.30	0.85	0.30
Methylisoeugenol cis	0.25	0.535	0.63	0.535	0.25
Methylisoeugenol trans	0.25	0.35	0.45	0.35	0.25
Dihydromethyleugenol	0.70	1.25	1.50	1.20	0.70
Safrole	0.20	#3.50	>5 00	1.15	0.15
Isosafrole cis	0.75	1.68	2.50	1.25	0.25
Isosafrole trans	0.50	1.28	1.75	1.13	0.50
Dihydrosafrole	1.50	3.45	5.70	2.18	1.00

All doses expressed in g/kg of mouse body weight.

TABLE II. Observations of temperatures a (new animals). Ambient temperature: $25 \pm 1^{\circ}$.

Temperatures a	Percentages
37.0 - 37.2	1.42
37.3 - 37.5	1.44
37.6 - 37.8	11.43
37.9 - 38.1	14.28
38.2 - 38.4	38.88
38.5 - 38.7	27.14
38.8 - 39.0	5.41

Table III.

Values of (b - a) (new animals). Ambient temperatures: 25 ± 1°.

Temperatures a considered	Values $\frac{(b-a)}{n}$	Extreme values of (b-a) Minima	Maxima
37.0 - 37.2	1.15	0.5	1.1
37.3 - 37.5	1.05	0.4	1.1
37.6 - 37.8	0.6	0.3	1.4
37.9 - 38.1	0.7	0	1.4
38.2 - 38.4	0.55	0	1.2
38.5 - 38.7	0.4	0	0.9
38.8 - 39.0	0.3	0	0.5

n indicates the population of the group having had the temperatures a shown.

TABLE IV.

Substances studied at a single dose of 0.10 g/kg.

Substance under study	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees) after 1 hour	after 6 hours
Cis-isoeugenol	4 in the 30th min.	3.7	1.2
Trans-isoeugenol	1.5 in the 20th min.	1.5	0
Eugenol	1 in the 40th min.	0.8	0
Dihydroeugenol	0.9 in the 45th min.	0.6	0

TABLE V.

Substances studied at equal doses in g/kg at half the infralethal dose.

Substance studied	Quantity used	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees); after 1 hours	after 6 hours
Cis-isoeugenol	0.05	1.9 in the 15th min	0.3	0
Trans-isoeugenol	0.10	1.5 in the 20th min	0	0
Eugenol	0.20	3.9 in the 70th min	3.7	1.3
Dihydroeugenol	0.25	3.7 in the 30th min	2.2	0

TABLE VI.

Substance studied	Index of aggressity (in g/kg)	Dose used (in g/kg)	Maximum observed temperature drop (in degrees)	Temperature drop after 1 hours	after 6 hours
Dihydroeugenol	0.55	0.50	8.6 in the 60th min.	8.6	2.2
Eugenol	0.40	0.35	5.4 in the 40th min.	5.2	1.4

Curve 1 Intreparitoneal injection of 0.20 g/kg of eugenol. Ordinates: temperature drops; Abscissae: Time

Curve 2 Intreperitoneal injection of 0.55 g/kg of dihydroeugenol. Ordinates: temperature drops; Abscissae: Time.

TABLE VII - Substances studied at the single dose of 0.10 g/kg.

Substance studied	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees); after 1 hours	after 6 hours
Cis-methylisoeugenol	2.0 in the 70th min.	1.0	0.9
Trans-methyl-isoeugenol	1.6 in the 75th min.	1.5	1.0
Methyleugenol	1.1 in the 85th min	0.8	5
Dihydromethyl-eugenol	0.9 in the 75th min	0.8	0

TABLE VIII - Substances studied at the single dose of 0.10 g/kg.

Substance studied	Quantity used (in %)	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees); after 1 hour	after 6 hours
Trans-methyliso-eugenol	0.10	1.6 in the 75th min	1.5	1.0
Cis-methyliso-eugenol	0.10	2.0 in the 70th min	1.5	1.0
Methyleugenol	0.15	1.8 in the 85th min	1.0	0.8
Dihydromethyl-eugenol	0.35	1.0 to the 30th min	0.9	0

TABLE IX

Substance studied	Quantity used	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees); after 1 hour	after 6 hours
Trans-methylisoeugenol	0.20	7.5 in the 270th min.	2.8	6.5
Cis-methylisoeugenol	0.20	6.7 in the 270th	2.4	5.5
Methyleugenol	0.35	5.3 in the 60thmin.	5.3	2.0
Dihydromethyleugenol	.55	5.6 in the 90th min	4.9	1.7

Curve 3 . Intraperitoneal injection of 0.20 g/kg of methyleugenol. Ordinates: Temperaturd drops; Abscissae: Time.

Curve 4. Intreperitoneal injection of 0.20 g/kg of cis-methylisoeugenol. In ordinates: Temperature drops; In Abscissae: Time.

TABLE X - Substances studied at the single dose of 0.10 g/kg

Substance studied	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees) after 1 hour	after 6 hours
Cis-isosafrole	5.0 in the 165th min.	3.7	3.8
Trans-isosafrole	2.6 in the 400th min.	0.4	2.4
Safrole	1.4 in the 200th min.	0.2	0
Dihydrosafrole	0.6 in the 120th min.	0	0

TABLE XI - Substances studied at equal doses in g/kg at half the infralethal dose.

Substance studied	Quantity used	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees) after 1 hour	after 6 hours
Trans-isoafrole	0.25	4.6 in the 315th min.	1.3	4.0
Dihydrosafrole	0.50	1.5 in the 120th min.	1.0	1.2

TABLE XII

Substance studied	Quantity used	Maximum observed temperature drop (in degrees)	Temperature drop (in degrees) after 1 hour	after 6 hours
Safrole	0.30	1.0 in the 260th min.	0.4	0.8
Cis-isosafrole	0.30	6.5 in the 240th min.	3.6	5.0
Trans-isosafrole	0.30	5.8 in the 240th min.	2.2	2.7

Curve 5. Intraperitoneal injection of 0.20 g/kg of cis-isosafrol. Ordinates: temperature drops; Abscissae: Time

Curve 6. Intraperitoneal injection of 0.35 g/kg of trans-isosafrol. Ordinates: temperature drops; Abscissae: Time.

Translated by Carl Demrick Associates, Inc./ARB/db

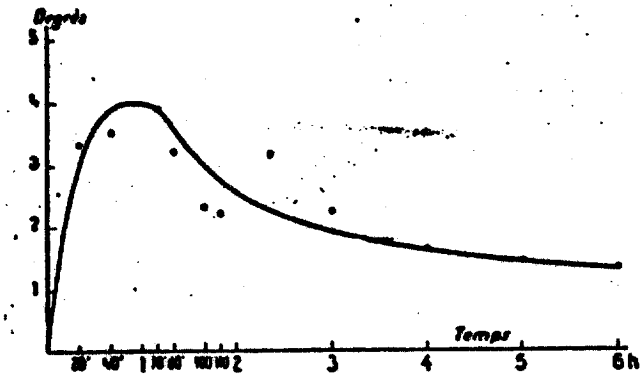

Fig. 1

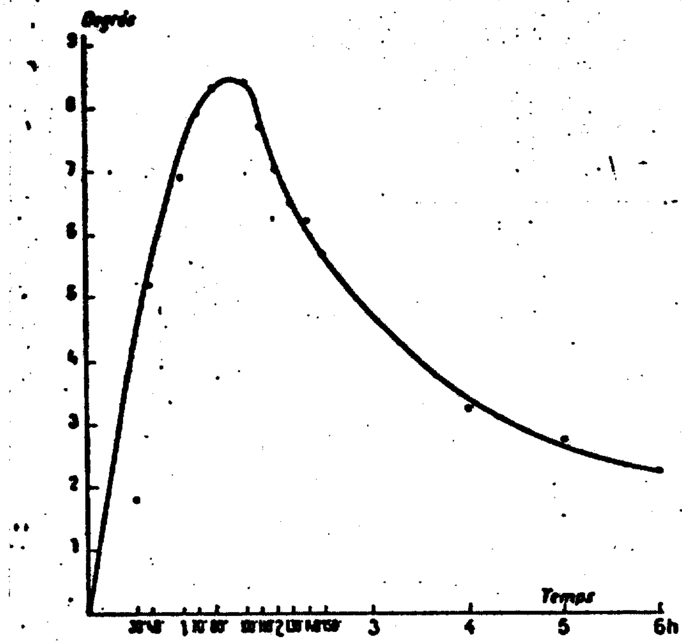

Fig. 2

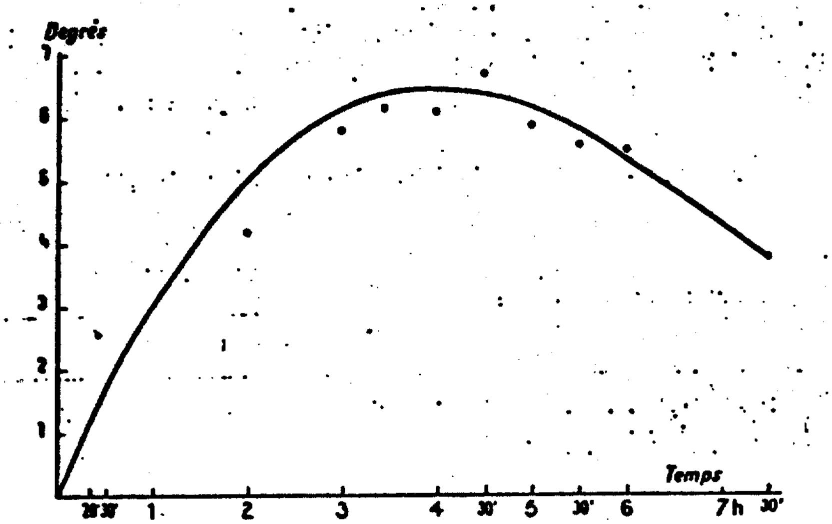

Fig. 3

Courbe 3. — Injection intrapéritonéale de 0,20 g/kg de méthyleugénol. *En ordonnées :* chutes thermiques. *En abscisses :* temps.

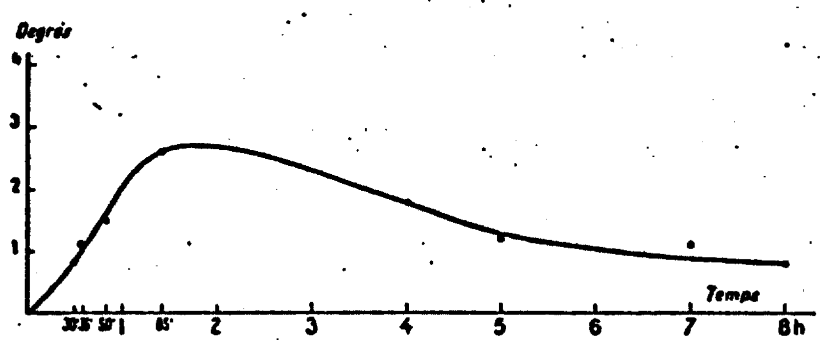

Fig. 4

PHARMACOLOGICAL STUDY OF ESSENTIAL OILS

Antispasmodic activity studied on some fifty different samples

by A. M. DEBELMAS and J. ROCHAT

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Essential oils, at all times used empirically for various therapeutic purposes, have often been the subject of experimental work. Their anti-microbial (4, 14, 18, 19, 22, 23, 27), cytophylactic (4, 11), narcotic properties in cold blooded animals (5, 12), or yet their power of percutaneous penetration (6, 26) have been studied in turn.

One of us had previously approached the question of their value as anthelmintics (7).

Now, in the course of recent research, one of the "in vitre" tests consisted in observing the activity of essential oils or their components on anterior segments of Ascaris lumbricoides mounted according to the technique of TOSCANO, RICO SILVIO REBELLO (25), which corresponds to that of MAGNUS using isolated organs. We were then struck by the inhibiting action of certain samples on spontaneous contractions of these preparations. This action could have occurred by means of the cephalic ganglions of the Ascaris, but also directly on the myofibrillae of this Nematode.

Thus we wished to resume this study on the smooth musculature of higher organiams in order to evaluate the possibilities of antispasmodic action in essential oils and also in an attempt to elucidate its mechanism of action. Thus since three years we have carried out the systematic study of some fifty essential oils and their components. Here we shall indicate only the results obtained with essential oils used in the formof saturated waters.

Little pharmacological work has been done in this direction. Let us mention however the clinical or experimental studies on isolated organs performed by MATCH (17), W. SALANT and C. W MITCHEL (24), Al. MUIRHEAD and H. F. GERALD (20) and K. BOETGE (3), and also those concerning certain components of these essences, such as the study of the anesthetic activity of terpenes and their semi-synthetic derivatives (1).

The part of the work reported on here concerns the influence of these substances with respect to the activity caused by the classical spasmogens on different isolated organs of mammals (ileon, duodenum, jejunum, seminal vesicles and aorta muscle).

EXPERIMENTAL METHOD

We used the general technique, derived from that of MAGNUS (16), making use of isolated organs.

- The animals are secrificed by carotid section (Rabbit) or by decapitation (Rat, Guinea Pig). The organs are removed and rid of their attached tissues. One of their extremities is held at a fixed point, the other is connected to an isotonic lever equipped with a scriber. The 20 ml survival physiological bath in which they are maintained is peculiar to each, with respect to ionic composition and temperature.

In this manner we used fragments of ileon of Guinea Pig, duodenum of Rat and jejunum of Rabbit maintained in Tyrode liquid at 37°, seminal vesicles of Rat in the liquid at 32° as prescribed by OHLIN and STROMBLAD (21) for the vas deferens and spiral shreds of aorta of Rabbit (10) maintained in a KREBS-HEINSEILET liquid (15) at 37°.

The choice of each of these organs was determined by the specificity and the faithfulness of their responses to the different spasmogens. Thus the various antagonistic actions were sought on the ileon of Guinea Pig with respect to histamine; on the duodenum of Rat for acetylcholine and barium chloride, on the siminal vesicles of Rat as well as on the aorta of Rabbit for adrenalin and finally on the jejunum of Rabbit for nicotine.

A first screening was performed on ileon, duodenum and seminal vesicles of the Rat. When an action was observed, the study was specified for the jejunum and aorta of the Rabbit.

Fromthe beginning we directed our work towards the observation of the antispasmodic activity of these substances by prior contact with the isolated organ.

The inhibiting effects observed were expressed in % of amplitude of response to the spasmogens used before contact with the saturated waters of essential oils They were compared with the actions obtained by means of classical antagonists (Promethazine Chlorhydrate, Atropine sulfate, Papaverin chlorhydrate, Ergotamine tartrate and Hexamethonium bromide). Each value cited is the average of at least three observations.

PREPARATIONS OF ESSENTIAL OILS USED

The essences were obtained from Etablissements CHIRIS, and each of them was characterized by its index of refraction and its density. Plate chromatographs and dosages of the principal constituents were prepared for the most active of them.

We are dealing with essentially liposoluble substances and we must provide a suitable contact with the isolated organ in order to make a valid observation of their action. This involves the use of homogeneous preparations.

Our attempts to use alcohol solutions were quickly abandoned, since alcohol has an action on certain organs. The same occured with dispersions made with non-ionic tensio-active agents (known to be the least toxic) whose antispasmodic action is far from negligible.

Nor can we use gummy Julep like for the study of these substances on segments of Ascaris, for the presence of saccharose in the survival baths of the organs then modifies their spontaneous activity.

At first, therefore, we studied only saturated waters of essential oils, which can be fairly satisfactory for a xreening, but does not make it possible in fact to compare the activities observed in view of the very low and different solubilities of the constituents. It must also be emphasized that, in these conditions, certain activities may not have been observed since the active principles may be non-soluble. These saturated waters were prepared according to the A. JONADET (13) technique, i.e. by contact for 24 hours between 1 ml of essential oil and 9 ml of physiological serum.

RESULTS

We have grouped in one table the interesting observations noted during this screening.

Inhibiting actions on the various smooth muscles

A. Animals

B. Rat

C. Guinea Pig

D. Rabbit

E. Organs

F. Seminal Vesicles

G. Duodenum

H. Ileon

I. Aorta

J. Jujunum

K. Spasmogens

L. Saturated Waters of Chenopode

M. Of Clove

N. Of Carvi

O. Of Sage

P. Of Thyme

Q. Of Melissa

Ad. = Adrenalin

Ac. Ch. = Acetylcholine	up to 20% inhibition		$\pm$
	from 20 to 40%	"	+
	from 40 to 60	"	++
	above		+++

Hi. = Histamine

Nic. = Nicotine

Examination of the results obtained shows that: 1) the different saturated waters of essential oils cited here possess a clear antihistamine action, comparable to that of doses of Promethazine Chlorhydrate near 0.01 to 0.02 ug.

2) All also proved active on nicotinic spasms, and the inhibitions obtained in thise case were, for the most active, near those produced by doses of 300 ug

of Hexamethonium Bromide.

3) The saturated water of essential oil of Thyme possesses a marked activity with respect to all the spasmogens used (8-9), resulting from a fairly general mechanism of action.

4) The saturated waters of essential oils of Chenopode and of Clove have an especially musculotropic spasmolytic activity, comparable to the effects obtained by means of 100 ur of Papaverin Chlorhydrate.

5) The saturated waters of essential oils of Melissa, Sage and Carvi exert their action essentially on the acetylcholinic spasm, which they inhibit in the same proportions as doses of 0.01 to 0.02 ug of Atropine, and thereby behave like neurotropic spasmolytics.

6) Antispasmodic actions with respect to Adrenalin are obtained much more rarely.

CONCLUSIONS

Despite the gaps mentioned during the discussion, and which are common to all screenings, this one gave us various interesting elements:

- General antispasmodic action of the soluble constituents of essence of Thyme Now it is known that Thyme is used in medicine in certain countries (12) as a sedative for cough spasms, of the whooping couth type.

- Musculotropic type antispasmodic action of the soluble constituents of essences of Clove and Chenopode.

- Marked neurotropic antispasmodic action of the soluble constituents of essences of Melissa, Sage and Carvi.

These differences of action are furthermore full of teaching inthemselves. In fact we were able to question the origin of the narcotic or microbicidal activity or of the power of percutaneous penetration of the essences by asking if these actions, fairly generall observed in this series of products, were not due to their physical properties (volatility, liposolubility, etc.). In the case of our study, in view of the different characteristics of the actions observed on the various smooth muscles studied, it can conceivably be stated that these action depend on different active principles. This is why we continued their study in order to pinpoint, if possible, the fractions responsible for the activities observed.

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Animaux A	Rat B			Cobaye C	Lapin D	
Organes E	Vésicules séminales F	Duodenum G	Duodenum G	Iléon H	Aorte I	Jejunum J
K Spasmogènes	Ad.	Ac. Ch.	$BaCl_2$	Hist.	Ad.	Nic.
L Eaux saturées de Chenopode	0	±	++	+++	0	+
de Girofle M	±	+	++	+++	±	+++
de Carvi N	0	+	±	+	0	+++
de Sauge O	0	+	0	+++	0	+
de Thym P	++	++	++	+++	++	+++
de Mélisse Q	0	++	±	++	+	++

Ad. = Adrénaline
Ac. Ch. = Acétylcholine
jusqu'à 20 % d'inhibition ... ±
de 20 à 40 % ... +
Hi. = Histamine
Nic. = Nicotine
de 40 à 60 % ... ++
au-dessus ... +++

35

The Application of $\Delta\epsilon$-Analysis to Pharmaceuticals: The Determination of Eugenol*

By JULIUS C. DEMETRIUS, Jr.†, and JOSEPH E. SINSHEIMER

The $\Delta\epsilon$-method of analysis, which was developed for the investigation of lignins, involves the selective modification of a given chromophore in a mixture of chromophores. This method permits the quantitative determination of a single ultraviolet-absorbing compound in mixtures of such absorbing materials. The analyses of eugenol in clove oil, in eugenyl acetate, and in two formulations are described as illustrations of the pharmaceutical application of this technique. The absorbance at 296 mμ of an aliquot of the eugenol-containing preparation was determined in basic solution. The change in absorbance was then compared to the bathochromic displacement of a standard eugenol solution with a corresponding change in pH.

THE STUDY of the phenolic compounds of various lignins by Aulin-Erdtman (1, 2) and by Goldschmid (3, 4) was based in part upon the development of "$\Delta\epsilon$-curves." In these investigations the $\Delta\epsilon$-curves were obtained by subtracting the absorbances of the U. V. spectra of lignin derivatives in solution of low pH from their corresponding spectra in solution of high pH. By comparing $\Delta\epsilon$-curves obtained in this manner to those obtained from model compounds in a similar manner, both qualitative and quantitative studies of phenolic compounds, even in the presence of other U. V. absorbing material, were possible.

The method need not be limited to the bathochromic displacement of phenols in alkaline solution. Aulin-Erdtman (5) in a review article points out that the $\Delta\epsilon$-method may be applied whenever the absorption properties of a given chromophore can be modified selectively in the presence of a mixture of chromophores. A $\Delta\epsilon$-curve is still obtained by subtracting the spectrum of the starting material from the spectrum of the product. The absorbance of any non-modified chromophores is thereby cancelled out. Thus, in those cases where selective modification of the chromophore can be accomplished, $\Delta\epsilon$-analysis has the advantage of the analysis of a single absorbing material in a mixture of absorbing materials without the necessity for prior separation.

This technique should have wide application to pharmaceutical determinations. It is the purpose of this investigation to apply $\Delta\epsilon$-analysis to a quantitative pharmaceutical problem. The lignin studies involved eugenol as one of the model compounds. A direct pharmaceutical extension of these studies was undertaken in the assay of clove oil for its eugenol content and the assay of eugenol in a pharmaceutical product. In addition, the procedure developed for the assay of clove oil was tested by its application to a sample of eugenyl acetate.

EXPERIMENTAL

Ultraviolet absorption spectra and quantitative measurements were made with a Beckman, model DU spectrophotometer. All quantitative measurements were the average of six determinations. After the first three determinations, the cells containing the sample solution and blank solution were interchanged.

Materials.—Eugenol (Eastman Kodak Co.) was fractionated by vacuum distillation through a helices-packed column. The refractive index of the purified sample was $n_D^{20} = 1.5410$. Eugenyl acetate (Aldrich Chemical Co.) was fractionated by a similar distillation and crystallized from alcohol-water. The melting point of the purified sample was 30-31°. Clove oil U. S. P. (Magnus, Mabee and Reynard, Inc.) had a total phenolic content of 87% as determined by the U. S. P. XV assay. All other materials were of C. P. grade and were used without further purification.

Determination of Eugenol Spectra.—An accurately weighed sample of 59 mg. of eugenol was diluted with 50% alcohol-water to 250 ml. in a volumetric flask. A series of 10-ml. portions of this solution was transferred to 100-ml. volumetric flasks and diluted to the mark with a series of buffer solutions prepared from standard solutions of sodium hydroxide and sulfuric acid. The pH of each of these eugenol solutions was measured by a Beckman Zeromatic pH meter and its U. V. spectrum determined. In this manner eleven spectra of eugenol in solutions ranging from pH 2.5 to 13.5 were obtained.

The Determination of $\Delta\epsilon$ for Eugenol.—Samples of about 50 mg. of eugenol were accurately weighed and diluted with alcohol in a 250-ml. volumetric flask. One 10-ml. portion of this solution was diluted with water and 1 ml. of 1.000 N sodium hydroxide in a 100-ml. volumetric flask. A second 10-ml. aliquot of the alcoholic eugenol solution was transferred to a 100-ml. volumetric flask and diluted with water and 1 ml. of 0.100 N sulfuric acid. The absorbance of the alkaline solution was determined relative to the acid solution in the ref-

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erence cell at 296 mμ. Blank solutions were also prepared, and any relative absorbance was determined in a like manner in order to correct for any contribution due to solvents and reagents. Seven such determinations were made. The alkaline solutions were recorded with a pH of 12.0 ± 0.2 and the acid solutions with a pH of 3.0 ± 0.2.

The Determination of Eugenol in Clove Oil and in Eugenyl Acetate.—Samples of about 60 mg. were accurately weighed and placed together with 3 ml. of 1 *N* sodium hydroxide and 15 ml. of alcohol in a 250-ml. volumetric flask. After the mixture was shaken for five minutes at room temperature, it was heated in a boiling water bath for fifteen minutes. During this time the flasks were shaken at five-minute intervals. The flasks were cooled to room temperature and alcohol was added to 250 ml. At the same time a control solution without sample was also prepared in this manner. Ten-milliliter aliquots of the saponified samples and the control were then diluted and buffered; the relative absorbances at 296 mμ were determined essentially as described under the determination of $\Delta\epsilon$ for eugenol. The only modification required was the addition of 2 ml. of 0.100 *N* sulfuric acid to obtain the desired pH of 3.0 ± 0.2 for the reference solution.

The Determination of Eugenol in Formulations.—An aliquot of the formulation was chosen to obtain a concentration of about 2 mg. per 100 ml. of eugenol in the reference and alkaline solutions. The procedure described under $\Delta\epsilon$ for eugenol was then followed.

Calculations and Definitions.—$\Delta\epsilon$ for eugenol: $\Delta\epsilon = (\Delta A \times 164.2)/c$

Percentage of eugenol: $\% = (\Delta A \times 164.2 \times 100)/(c \times \Delta\epsilon)$

Concentration: $c = (\Delta A \times 164.2)/\Delta\epsilon$

Where: $\Delta\epsilon$ = absorbance at 296 mμ of a 1*M* solution of eugenol in base less the absorbance of a 1*M* solution in acid. ΔA = the observed absorbance at 296 mμ of a given concentration of eugenol in basic solution less the absorbance of same concentration of eugenol in acid solution. c = concentration in Gm./L.

RESULTS AND DISCUSSION

Figure 1 represents the spectra of eugenol in (*a*) acid to neutral solution and (*b*) in alkaline solution. As is typical of phenols, there is a pronounced bathochromic displacement of both the E and B bands and an increase in the intensity of absorption of these bands. When the acid spectra is subtracted from that of the alkaline spectra, the $\Delta\epsilon$-curve illustrated in Fig. 2 is obtained.

The same $\Delta\epsilon$-curve is produced even in the presence of other U. V.-absorbing compounds provided that these compounds show no response to changes in pH. $\Delta\epsilon$-curves can be used in a manner analogous to normal U. V. curves for qualitative and quantitative purposes. Thus, the maxima at 246 and 296 mμ should serve as the basis for quantitative measurements.

It appears that the 246 mμ maximum, because of its greater intensity, would be the most useful for quantitative measurements. However, in actual

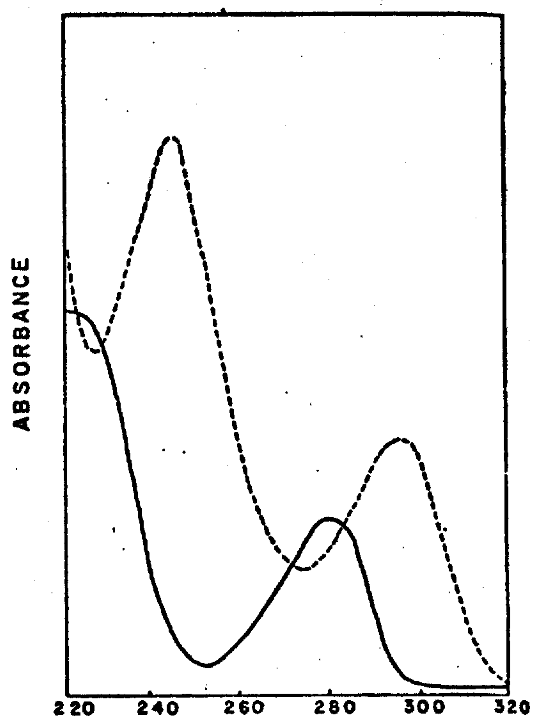

Fig. 1.—Ultraviolet absorbance spectra of eugenol; —— pH 7.0; - - - - pH 12.3.

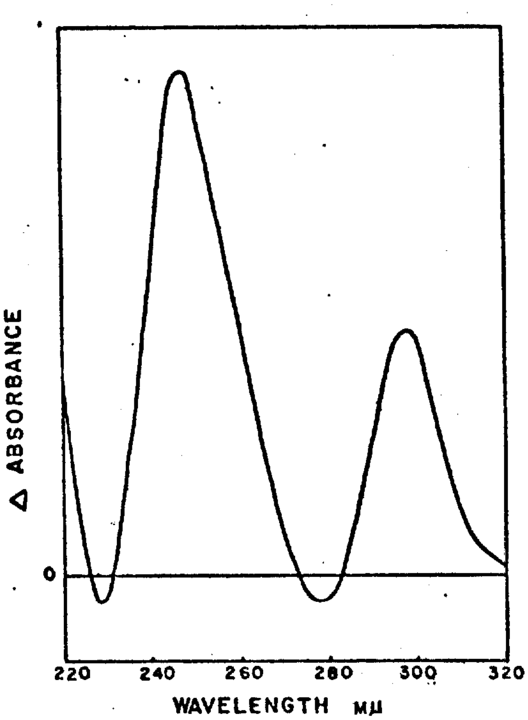

Fig. 2.—$\Delta\epsilon$ ultraviolet absorbance spectrum of eugenol at pH 12.3 with the eugenol spectrum at pH 7.0 as a reference.

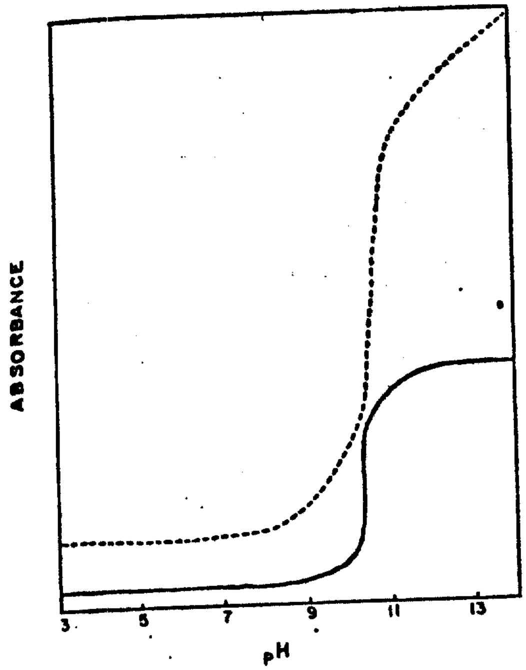

Fig. 3.—Maximum absorbance of eugenol as a function of pH; —— maximum at 296 mμ; - - - - maximum at 246 mμ.

practice this is not the case, since the 296 mμ maxima showed greater stability with changes in alkaline pH than did the 246 mμ maxima and, therefore, would require a less critical control of pH for quantitative comparisons. The change in absorbance of these peaks as a function of change in pH is illustrated in Fig. 3.

To serve as a basis for the quantitative comparison of eugenol solutions, the $\Delta\epsilon$ for the 296 mμ maxima was determined from standard solutions. The average of seven determinations gave a value of 3,886 for a molar solution of eugenol of a pH of 12.0 ± 0.2 over that of a molar solution of a pH of 3.0 ± 0.2 at 296 mμ.

Table I is a summary of the results of the determination of the percentage of eugenol in a sample of clove oil based upon this value for $\Delta\epsilon$. As a partial test of the procedure there is also included in Table I the results of the analysis of a sample of eugenyl acetate.

In order to investigate the application of $\Delta\epsilon$-analysis to a pharmaceutical formulation the following mouth wash, formula A, was compounded.

Saccharin, soluble................	0.025 Gm.
Fuchsin, basic..................	0.005 Gm.
Peppermint oil..................	0.100 Gm.
Eugenol........................	0.100 Gm.
Alcohol........................	75.00 cc.
Distilled water to..............	250.00 cc.

Table II presents the results of the analysis of this solution for its eugenol content.

TABLE I.—ANALYSIS OF EUGENOL IN CLOVE OIL AND EUGENYL ACETATE

Sample	Clove Oil, %	Eugenyl Acetate, % (79.6% Calcd.)
1	85.4	80.0
2	83.8	79.9
3	85.7	81.2
4	86.7	79.5
5	86.0	...
6	84.9	...
7	85.8	...
Av.	85.5	80.2

TABLE II.—ANALYSIS OF EUGENOL IN MOUTH WASH FORMULAS

	Formula A, Gm.	Formula B, Gm.
Amount present	0.1004	0.1124
Found 1	0.0980	0.1090
2	0.0980	0.1107
3	0.0997	0.1104
4	0.0976	0.1101
5	0.1001	0.1094
6	0.0972	0.1103
Av.	0.0984	0.1099

The above formula was also modified by reducing the peppermint oil to 0.06 Gm. and including 0.06 Gm. of cassia oil which possesses U. V. absorption of high intensity. The results for the analysis of eugenol in the modified formula, formula B, are also presented in Table II.

It should be noted that the U. V. spectrum of cassia oil shows a limited sensitivity to changes in pH, and results recorded in Table II would have been about 10% lower unless corrected for this change. That is, the analysis of formula B required that the absorbance of a control solution without eugenol also be determined and be applied as a correction to the analysis of the complete formula. Formula B, therefore, demonstrates a limitation of $\Delta\epsilon$-analysis: the sensitivity of the other compounds in a mixture to the conditions used to modify the chromophore of interest. This limitation can be overcome in the analysis of dosage forms by running a control as noted in the analysis of formula B or by applying a simultaneous equation determination to the $\Delta\epsilon$-curve. If such corrections cannot be applied as, for example, in the assay of some natural products, the extent of these errors can readily be determined and, to a great degree, corrected by comparing the complete $\Delta\epsilon$-spectrum of mixture to that of the standard compound.

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SCIENTIFIC SECTION
ESSENTIAL OIL ASSOCIATION OF U. S. A.
220 Fifth Avenue, New York 1, N. Y.

E.O.A. STANDARD for: OIL CLOVE LEAF

Oil Clove Leaf is the volatile oil distilled by means of steam from the leaves of the Clove tree, an evergreen belonging to the family—Mytacae, cultivated chiefly in Madagascar, the Seychelles, Zanzibar and Pemba. The tree is also cultivated on a smaller scale in other areas such as Reunion, Penang, and Amboina.

SPECIFICATIONS

Botanical Nomenclature	Eugenia caryophyllata, Thurnberg (Caryophyllus aromaticus, Linné).
Preparation	Obtained by steam distillation of the leaves.
Physical & Chemical Properties	Color & Appearance: Very pale yellow when freshly distilled, but discolors rapidly to a brown or even a purple shade when in contact with iron containers.
	Specific Gravity @ 25°/25°C: 1.036 to 1.046. (Correction factor from n°/n°: .00067 per °C.)
	Optical Rotation: 0° to –2°.
	Refractive Index @ 20°C: 1.5310 to 1.5350.
	Solubility in Alcohol: Soluble in 2 volumes of 70% alcohol; a slight opalescence often occurs when additional solvent is added.
	Heavy Metals: Negative to slightly positive. Proceed as directed under heavy metals test (See Determinations E.O.A. No. 1-L). Not more than a slight darkening in color is produced in either the oil or the water.
	Total Eugenol by Volume: 84% to 88%. Method: Place about 2% of powdered tartaric acid in the oil and shake thoroughly for about 2 minutes and filter. Then place 10-cc. of the filtered oil, measured from a pipette, in a 100-cc. cassia flask, add 75-cc. of 1 N potassium hydroxide solution, shake the mixture for 5 minutes then heat it for 10 minutes in a bath containing boiling water. Remove it from the bath, and cool to room temperature. When the liquids have separated completely add sufficient 1 N potassium hydroxide to raise the lower limit of the oily layer within the graduated portion of the neck. The volume of the insoluble oil in cubic centimeters is then subtracted from 10. The difference multiplied by 10 gives the percentage of Eugenol in the oil.

(OVER)

SPECIFICATIONS (Continued)

Descriptive Characteristics	**Solubility:**
	Benzyl Benzoate: Soluble in all proportions.
	Diethyl Phthalate: Soluble in all proportions.
	Fixed Oils: Soluble; often with slight opalescence in most fixed oils.
	Glycerine: Relatively insoluble.
	Mineral Oil: Relatively insoluble.
	Propylene Glycol: Soluble in all proportions.
	Stability:
	Alkali: Unstable.
	Acids: Fairly stable in presence of most dilute acids.
Containers	Should be shipped preferably in glass, tin lined, stainless steel, or aluminum containers. When galvanized containers are used a precipitate may result.
Storage	Store in full, tight containers in a cool place, protected from light.

SCIENTIFIC SECTION
ESSENTIAL OIL ASSOCIATION OF U. S. A.
2 Lexington Ave., New York 10, N.Y.

E.O.A. STANDARD for: OIL CLOVE STEM

Oil Clove Stem differs from the oil obtained from the Clove Buds. The odor, flavor and chemical composition differs from the Bud oil and is less acceptable for use in flavors and perfumes. It can be used for the isolation of good quality eugenol. Zanzibar is the principal source for this oil, small quantities are produced in other areas.

SPECIFICATIONS

Botanical Nomenclature	*Eugenia Caryophyllus* (Sprengel) *Bullock et Harrison* (formerly Eugenia Caryophylata Thunberg) or (Caryophyllus aromaticus L.). (Family: *Myrtaceae.)*
Preparation	Usually obtained by steam distillation of the dried stems after removal of the buds.
Physical and Chemical Constants	Appearance and Odor: Yellow to light brown liquid. In contact with iron the oil acquires a purplish dark brown shade. It has the characteristic clove spice odor but less pleasant than the oil from the buds. Specific Gravity at 25°/25°C: 1.048 to 1.056. (Temperature correction factor from n°/n°C = 0.0006 per °C.). Optical Rotation: ±0° to —1°30°. Refractive Index at 20°C: 1.5340 to 1.5380. Total Phenols Content: 89% to 95%. Method: Proceed as directed for the determination of Total Phenols. (See EOA Determinations #1-S). Solubility in Alcohol: Soluble in two and more volumes of 70% alcohol.
Descriptive Characteristics	Solubility: Benzyl Benzoate: Soluble in all proportions. Diethyl Phthalate: Soluble in all proportions. Fixed Oils: Soluble in all proportions in most fixed oils. Glycerine: Relatively insoluble. Mineral Oil: Relatively insoluble. Propylene Glycol: Soluble in all proportions. Stability: Acids: Fairly stable in the presence of weak organic acids. Alkali: Unstable.
Containers	Should be shipped preferably in glass, aluminum or tin-lined containers. A precipitate may result if galvanized iron containers are used.
Storage	Store in tight full containers in a cool place protected from light.

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SCIENTIFIC SECTION
ESSENTIAL OIL ASSOCIATION OF U.S.A.

2 Lexington Ave., New York, N. Y. 10010

E.O.A. STANDARD for: OLEORESIN CLOVES

Oleoresin cloves is the product obtained by solvent extraction of the dried flower buds of Eugenia caryophyllata Thumberg (Family Myrtaceae) with the subsequent removal of the solvent.

SPECIFICATIONS

Botanical Nomenclature	Eugenia caryophyllata Thumberg (Family Myrtaceae)
Preparation	Obtained by solvent extraction of the dried flower buds of cloves with subsequent removal of the solvent.
Physical and Chemical Constants . .	Appearance and Odor: A dark green, somewhat viscid, non-homogeneous liquid with the characteristic odor and flavor of Oil of Cloves. ~~Specific Gravity at 25°/25°C: 1.040 to 1.060.~~ Volatile Oil Content: 66 to 88 ml/100 gms. Method: Proceed as directed for the determination of volatile oil (See E.O.A. Determination of No. 57-G) using 10 gms. of sample accurately weighed and report as ml per 100 gms. of oleoresin. Optical Rotation of Oil: Not exceeding −1°30″. Refractive Index of Oil at 20°C: 1.5270 to 1.5380. Residual Solvent in Oleoresin: Meets with Federal Food, Drug & Cosmetic Act Regulations.
Descriptive Characteristics	Solubility: Alcohol: Partly soluble with oil separation and sediment. Benzyl Benzoate: Soluble in all proportions. Fixed Oils: Soluble in all proportions with sediment in most fixed oils. Glycerine: Insoluble. Mineral Oil: Partly soluble with oil separation and sediment. Propylene Glycol: Partly soluble with oil separation and sediment.
Containers	Ship in glass or suitably lined containers.
Storage	Store preferably in full, tight containers in a cool place, protected from light.

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Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 772, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in full, tight, preferably glass, tin-lined or other suitably lined containers in a cool place protected from light.

Functional use in foods. Flavoring agent.

CLOVE LEAF OIL

DESCRIPTION

The volatile oil obtained by steam distillation of the leaves of *Eugenia caryophyllata* Thunberg (*Eugenia aromatica* (L.) Baill.). (Fam. *Myrtaceae*). It is a pale yellow liquid. It is soluble in propylene glycol, in most fixed oils, with slight opalescence, and relatively insoluble in glycerin and in mineral oil.

SPECIFICATIONS

Assay. Not less than 84 per cent and not more than 88 per cent, by volume, of phenols as eugenol ($C_{10}H_{12}O_2$).

Angular rotation. Between −2° and 0°.

Refractive index. Between 1.5310 and 1.5350 at 20°.

Solubility in alcohol. Passes test.

Specific gravity. Between 1.036 and 1.046.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 per cent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 per cent).

Lead. Not more than 10 parts per million (0.001 per cent).

TESTS

Assay. Shake a suitable quantity of the oil with 2 per cent of powdered tartaric acid for about 2 minutes and filter. Then, using a sample of the filtered oil, proceed as directed under *Phenols*, page 745, modified by heating the flask in a boiling water bath for 10 minutes, after shaking the oil with potassium hydroxide T.S. Remove from the boiling water bath, cool, and proceed as directed.

Angular rotation. Determine in a 100-mm. tube as directed under *Optical Rotation*, page 780.

Refractive index, page 785. Determine with an Abbé or other refractometer of equal or greater accuracy.

Solubility in alcohol. Proceed as directed in the general method, page 746. One ml. dissolves in 2 ml. of 70 per cent alcohol. A slight opalescence may occur when additional solvent is added.

Specific gravity. Determine by any reliable method (see page 4).

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 720.

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 772, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in full, tight, light-resistant, glass, tin-lined, stainless or aluminum containers in a cool place protected from light.

Functional use in foods. Flavoring agent.

CLOVE OIL

DESCRIPTION

The volatile oil obtained by steam distillation from the dried flower-buds of *Eugenia caryophyllata* Thunberg (*Eugenia aromatica* (L.) Baill.). (Fam. *Myrtaceae*). It is a colorless or pale yellow liquid having the characteristic clove odor and taste. It darkens and thickens upon aging or exposure to air.

SPECIFICATIONS

Assay. Not less than 85 per cent, by volume, of phenols.

Angular rotation. Between −1° 30′ and 0°.

Refractive index. Between 1.5270 and 1.5350 at 20°.

Solubility in alcohol. Passes test.

Specific gravity. Between 1.038 and 1.060.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 per cent)

Heavy metals (as Pb). Not more than 40 parts per million (0.004 per cent).

Lead. Not more than 10 parts per million (0.001 per cent).

Phenol. Passes test.

TESTS

Assay. Proceed as directed under *Phenols*, page 745.

Angular rotation. Determine in a 100-mm. tube as directed under *Optical Rotation*, page 780.

Refractive index, page 785. Determine with an Abbé or other refractometer of equal or greater accuracy.

Solubility in alcohol. Proceed as directed in the general method, page 746. One ml. dissolves in 2 ml. of 70 per cent alcohol.

Specific gravity. Determine by any reliable method (see page 4).

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test,* page 720.

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test,* page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test,* page 772, using 10 mcg. of lead ion (Pb) in the control.

Phenol. Shake 1 ml. of sample with 20 ml. of hot water. The water shows no more than a scarcely perceptible acid reaction with blue litmus paper. Cool the mixture, pass the water layer through a wetted filter, and treat the clear filtrate with 1 drop of ferric chloride T.S. The mixture has only a transient grayish green color, but not a blue or violet color.

Packaging and storage. Store in full, tight, light-resistant containers and avoid exposure to excessive heat.

Functional use in foods. Flavoring agent.

CLOVE STEM OIL

DESCRIPTION

The volatile oil obtained by steam distillation from the dried stems of the buds of *Eugenia caryophyllata* Thunberg (*Eugenia aromatica* (L.) Baill.). (Fam. *Myrtaceae*). It is a yellow to light brown liquid with a characteristic odor and taste. It is soluble in fixed oils, and in propylene glycol, but it is relatively insoluble in glycerin and in mineral oil.

SPECIFICATIONS

Assay. Not less than 89 per cent and not more than 95 per cent, by volume, of phenols as eugenol ($C_{10}H_{12}O_2$).

Angular rotation. Between −1.5° and 0°.

Refractive index. Between 1.5340 and 1.5380 at 20°.

Solubility in alcohol. Passes test.

Specific gravity. Between 1.048 and 1.056.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 per cent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 per cent).

Lead. Not more than 10 parts per million (0.001 per cent).

TESTS

Assay. Shake a suitable quantity of the oil with about 2 per cent of powdered tartaric acid for about 2 minutes and filter. Then, using a sample of the filtered oil, proceed as directed under *Phenols*, page 745, modified by heating the flask in a boiling water bath for 10 minutes, after shaking the oil with potassium hydroxide T.S. Remove from the boiling water bath, cool, and proceed as directed.

Angular rotation. Determine in a 100-mm. tube as directed under *Optical Rotation*, page 780.

Refractive index, page 785. Determine with an Abbé or other refractometer of equal or greater accuracy.

Solubility in alcohol. Proceed as directed in the general method, page 746. One ml. dissolves in 2 ml. of 70 per cent alcohol.

Specific gravity. Determine by any reliable method (see page 4).

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 720.

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 772, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store preferably in full, tight, light-resistant glass, aluminum, or tin-lined containers in a cool place protected from light.

Functional use in foods. Flavoring agent.

COGNAC OIL, GREEN

Wine Yeast Oil

DESCRIPTION

The volatile oil obtained by steam distillation from wine lees. It is a green to bluish green liquid with the characteristic aroma of cognac. It is soluble in most fixed oils and in mineral oil. It is very slightly soluble in propylene glycol, and it is insoluble in glycerin.

f

... to 1.055
... & to 88%

ds (I), three oils from Mauritius
nd (IV), examined by Schimmel

II	III	IV
1 to 1.067	1.0535	1.0462
50′ to −1° 0′	−0° 49′	−1° 20′
63 to 1.5391	1.53513	1.53244
o 93%	90%	86%
arly soluble in vol. and more	Soluble in 1 vol. and more	Soluble in 0.9 vol. and more

to identify in clove bud oil are
ed clove leaf oil, the addition of
nly slightly. To prove sophisti-
is not easy and, therefore, one
expert will, in most cases, have
oil by its somewhat harsh note.
the addition of clove terpenes,
f eu[illegible]l from clove oil. It is
tities, but the addition of larger
lowers the eugenol content, the
the oil.
dibenzyl ether is best detected
g for a few days or, even better,
e oil. Acetins, too, are occasion-
nd can be detected by washing
gh saponification number of the
ins (see Vol. I of this work, p.

gn to clove oil is seldom encoun-
nonphenolic portions of the oil

54. Cf. Gildemeister and Hoffmann,

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should always be made; if purity is doubtful, a large sample should be freed from eugenol and the remaining nonphenolic portions fractionated *in vacuo* with a careful examination of each fraction.

The detection of water soluble adulterants can be effected by determining any change in properties before and after washing the oil with saturated salt solutions.

The most important test in the analysis of clove oil is the determination of the total phenols (chiefly eugenol)—see above, section on "Physicochemical Properties."

Chemical Composition

A. Clove Bud Oil.—The volatile oil derived from dried clove buds by distillation contains, as its main constituents, free eugenol (70 to 90 per cent), eugenol acetate, and caryophyllene. Although these substances amount to some 99 per cent of the oil, they are not, as Smith [33] has pointed out, responsible for the characteristic fresh and almost fruity note of a pure clove bud oil. The proof of this lies in the fact that it is only necessary to prepare a mixture of pure clove eugenol, eugenol acetate, and caryophyllene in correct proportions, and to compare it with a sample of natural clove bud oil. This is one of the many examples of the importance of those substances which are found only in traces in natural essential oils. Clove bud oil contains several of these substances, the most important one being methyl-*n*-amyl ketone. As a characteristic feature it should also be mentioned here that clove bud oil contains a substantial percentage of eugenol acetate, whereas clove stem oil and clove leaf oil contain only traces of it.

The following compounds [34] have been identified in clove bud oil:

Eugenol. The chief constituent of clove bud oil, constituting from 70 per cent to more than 90 per cent in free form.

Eugenol Acetate (Aceteugenol, Acetyl Eugenol). See above. Erdmann [35] reported 2 to 3 per cent, Spurge [36] 7 to 17 per cent, Smith [37] 10 to 15 per cent of eugenol acetate in clove bud oil.

Caryophyllene. This sesquiterpene was first noted in clove oil by Church; [38] later it was named caryophyllene by Wallach.[39] According to Gildemeister and Hoffmann,[40] it occurs in clove oil chiefly as lower boiling, laevorotatory β-caryophyllene

[33] *Perfumery Essential Oil Record* 37 (1946), 144.
[34] Details regarding these substances will be found in Vol. II of this work.
[35] *J. prakt. Chem.* [2], 56 (1897), 143.
[36] *Pharm. J.* 70 (1903), 701, 757. According to Spurge, the method of assay used by Erdmann is faulty.
[37] *Perfumery Essential Oil Record* 37 (1946), 144.
[38] *J. Chem. Soc.* 28 (1875), 113.
[39] *Liebigs Ann.* 271 (1892), 287.
[40] "Die Ätherischen Öle," 3d Ed., Vol. III, 286.

(blue nitrosite m. 164°–165°) and, in smaller proportion, as higher boiling, optically inactive α-caryophyllene (nitrosochloride m. 177°). According to Naves,[41] the volatile oil derived from cloves by steam distillation contains from 5 to 12 per cent of α- and β-caryophyllene, whereas the oil extracted from the spice with volatile solvents contains almost none.

Caryophyllene Oxide (Caryophyllene Epoxide). As far back as 1912, Vielitz[42] had noted a strongly laevorotatory hydrocarbon in the highest boiling fractions of clove oil, but it was only recently that Treibs[43] actually isolated caryophyllene oxide from clove oil. The biological parent substance of this oxide is undoubtedly caryophyllene. Treibs[44] reported these properties for caryophyllene oxide:

m.	64°
d_4^{20}	0.9658
α_D^{20}	−68° 0′
n_D^{20}	1.4958

According to a more recent publication of Naves,[45] the (benzene) extract of clove buds does not contain caryophyllene, but epoxy-dihydrocaryophyllene $C_{15}H_{24}O$ (the caryophyllene oxide of Treibs). However, the extracted mass of clove buds, under the influence of boiling water yields an essential oil composed chiefly of caryophyllene. The latter, therefore, is not a natural, biological constituent of the clove buds.

The epoxy-dihydrocaryophyllene observed by Naves in the (benzene) extract of clove buds had these properties:

m.	63°–64°
$b_{1.8}$	114°–117°
d_4^{20}	0.966
$[\alpha]_D$	−70° 2′
n_D^{20}	1.49564

As concerns those important substances that occur only in traces in clove bud oil, the following have been reported:

Methyl Salicylate. By Masson.[46]

Methyl-*n*-amyl Ketone. This compound, which is particularly responsible for the peculiar, almost fruity by-odor of the clove bud oil, was identified in the laboratories of Schimmel & Co.[47] through its semicarbazone m. 122°–123°, and through oxidation into valeric and acetic acids.

Methyl Alcohol, Furfural, β-Pinene(?), and Methyl Benzoate. In the course of their investigations the Schimmel chemists[48] isolated traces of still other substances from clove bud oil: methyl alcohol, furfural, β-pinene(?), and methyl benzoate.

[41] *Helv. Chim. Acta* **31** (1948), 378. Cf. Naves and Perrottet, *ibid.* **24** (1941), 790. Deussen, *Liebigs Ann.* **356** (1907), 1.
[42] *Inaugural Dissertation, Leipzig* (1912).
[43] *Chem. Ber.* **80**, No. 1 (1947), 56.
[44] *Ibid.*
[45] *Helv. Chim. Acta* **31** (1948), 380.
[46] *Compt. rend.* **149** (1909), 795.
[47] *Ber. Schimmel & Co.*, April (1897), 50; April (1902), 44.
[48] *Ibid.*, October (1896), 57; April (1903), 51, 52.

Methyl-*n*-heptyl Ketone. Identified through its semicarbazone m. 118°–119°.

Valeraldehyde(?). According to Schimmel & Co. the oil contains probably valeraldehyde.

Further work on the minor constituents of clove bud oil was carried out by Masson [49] who noted the presence of these compounds:

Methyl-*n*-amyl Carbinol (2-Heptanol). B. 157°–158°, d_0 0.8344. Isolated from the fraction b_{15} 50°–75° by treatment with phthalic anhydride. Oxidation of the carbinol gave methyl-*n*-amyl ketone b. 151°–152°.

Furfuryl Alcohol. The treatment of the fraction b_{15} 50°–75° with phthalic anhydride yielded also furfuryl alcohol b. 170°–171°, d_0 1.1615, which was identified through its diphenylurethane m. 97.5°.

α-Methyl Furfural. The fraction b_{15} 65°–95° contains (aside from methyl-*n*-heptyl ketone—see above) α-methyl furfural b. 184°–186°, b_{20} 75°, d_0 1.1365, which Masson identified through its semicarbazone m. 210°–211°, its phenylhydrazone m. 147°–148°, and through oxidation to α-methyl-pyromucic acid m. 107°–108°.

Methyl-*n*-heptyl Carbinol (2-Nonanol) and Benzyl Alcohol. In the fraction b_{15} 75°–100° Masson observed methyl-*n*-heptyl carbinol b. 195°–196°, d_0 0.8399, which was characterized by oxidation to methyl-*n*-heptyl ketone, and benzyl alcohol b. 206°, d_0 1.0627, which was oxidized to benzoic acid.

Methyl Furfuryl Alcohol(?). The same fraction of the oil probably also contains methyl furfuryl alcohol.

A Dimethyl Furfural(?). In the fraction b_{15} 105°–120° Masson reported a dimethyl furfural b. 206°–208°, which on oxidation yielded a pyromucic acid m. 129°–130°. It was not possible, however, to determine the position of the methyl groups in the molecule.

Vanillin. This aromatic aldehyde is most probably present in clove bud oil, at least in traces, and undoubtedly originates by air-oxidation of eugenol. According to Gildemeister and Hoffmann,[50] dried clove buds contain vanillin. Jorissen and Hairs [51] observed vanillin in clove oil. Shaking clove oil with (1/5 of its volume) sodium hydroxide solution, van Urk [52] extracted crystals m. 90°–92° which had a vanillin-like odor and which, on treatment with phloroglucinol and hydrochloric acid, developed a red color.

B. Clove Stem Oil.—The chemical composition of the oil derived from clove stems has not been investigated as thoroughly as that of the commercially much more important clove bud oil, which is used widely in food products and in pharmaceuticals. Literature on the subject agrees that in general the chief constituents present in clove bud oil occur also in the stem oil, but in somewhat different proportions. The percentage of *free eugenol* present in the stem oil, for example, is usually somewhat higher than that present in the bud oil. The stem oil, on the contrary, contains only a small

[49] *Compt. rend.* **149** (1909), 630, 795.
[50] "Die Ätherischen Öle," 3d Ed., Vol. III, 288.
[51] *Chem. Zentr.* (1890), II, 828.
[52] *Pharm. Weekblad* **65** (1928), 345.

amount of *eugenol acetate,* whereas the bud oil has been reported to contain up to 17 per cent of this ester. Among the other constituents present in clove stem oil, *α-* and *β-caryophyllene, furfural,* and *methyl alcohol* have been identified by the same workers who concerned themselves chiefly with the bud oil (see "Chemical Composition of Clove Bud Oil").

Substances, occurring only in traces, which impart the characteristic, almost fruity odor to the bud oil, seem to occur in the stem oil in still more minute quantities, or to be lacking entirely, which explains the coarser and "flatter" odor of the stem oil. Judging from the odor, *methyl-n-amyl ketone* appears to be present in clove stem oil but in a smaller proportion than in the bud oil.

On the other hand, clove stem oil contains a few constituents which have not yet been observed in clove bud oil. Von Soden and Rojahn[53] isolated traces of *naphthalene* from clove stem oil. Semmler and Mayer[54] observed an apparently bicyclic *sesquiterpene alcohol,* $C_{15}H_{26}O$, containing one double bond, which had these properties: b_8 138°–148°, d_{20} 0.9681, α_D −17°, n_D 1.5010, Mol. Refr. found 68.18, calculated for $C_{15}H_{26}O$ |‾68.07. When treated with alcoholic potassium hydroxide, the hydrochloride b_{12} 147°–155°, d_{20} 0.990 of this sesquiterpene alcohol yielded a hydrocarbon b_{10} 123°–126°, d_{20} 0.9273, α_D^{20} −23°, n_D^{20} 1.5024.

Fractionating the sesquiterpene portions of clove stem oil *in vacuo* (after removal of eugenol), Deussen[55] isolated from the distillation residue about 0.1 per cent of an amorphous, alcohol-insoluble substance m. 146° (not sharp), to which he assigned the empirical molecular formula $(C_{21}H_{30}O)_5$.

C. Clove Leaf Oil.—What has already been said about the chief constituents and the trace substances present in clove stem oil applies equally well to the oil derived from clove leaves. The chemical composition of clove leaf oil, like that of the stem oil, has not yet been as thoroughly investigated as that of the bud oil. Clove leaf oil usually contains a somewhat lower percentage of total eugenol than is present in clove bud oil; eugenol acetate occurs in the leaf oil, as in the stem oil, only in very small quantities. The trace substances, methyl-*n*-amyl ketone for example, which impart the characteristic, almost fruity odor to the bud oil, occur in the leaf oil probably in even more minute quantities than in the stem oil; hence the much coarser and "flatter" odor of the leaf oil.

As far as the substances which occur in the stem oil (but not in the bud oil) are concerned, viz., a sesquiterpene alcohol $C_{15}H_{26}O$, and naphthalene, only the latter (traces) has been observed in clove leaf oil.[56]

[53] *Pharm. Ztg.* **47** (1902), 779.
[54] *Ber.* **45** (1912), 1392.
[55] *Ibid.* **42** (1909), 380, 680.
[56] *Ber.-Schimmel & Co.* (1939), 57.

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Fd Cosmet. Toxicol. Vol. 5, pp. 141–157. Pergamon Press 1967. Printed in Great Britain

Research Section

Food Flavourings and Compounds of Related Structure. II. Subacute and Chronic Toxicity

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(*Received 7 November 1966*)

Abstract—The subacute and/or chronic toxicity of 48 food flavourings was studied in rats. The toxicity of five of these flavourings (coumarin, 6-methylcoumarin, dihydrocoumarin, methyl salicylate and safrole) was also studied in dogs. A summary of the studies is presented. The loss of 21 flavourings from laboratory animal diets during a 7-day period was also determined.

INTRODUCTION

For a number of years the Food and Drug Administration has been investigating the toxicity of food flavourings, both natural and synthetic. The toxicity studies consist of (1) determination of the acute oral effects, (2) subacute studies in which the flavouring agents are mixed in the diet or administered by stomach tube, and (3) chronic feeding studies on those flavouring agents having wide use, having chemical structures implying toxicity and/or showing toxic effects in the subacute studies. The data on the acute oral toxicity of these substances have been reported (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The present report is a summary of the subacute and chronic studies. Full reports on some of the chronic studies have been published for methyl salicylate (Webb & Hansen, 1963), safrole (Long, Nelson, Fitzhugh & Hansen, 1963; Hagan, Jenner, Jones, Fitzhugh, Brouwer & Webb, 1965) and dihydrosafrole (Long & Jenner, 1963; Hagan *et al.* 1965).

Commercially-available materials, rather than pure chemicals, were used since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives.

EXPERIMENTAL

Rat studies. Weanling Osborne-Mendel rats were used in the chronic and most of the subacute experiments. In a few subacute experiments young adult rats of the same strain were used. Litter mates were used except for a few subacute studies in which the weanlings were randomized by weight (every level having animals of equal weight). A control group containing the same number of rats as the test groups was included with each test compound except in the following studies in which a common group was used for a number of test compounds:

(1) Allyl caproate, allyl cyclohexane propionate, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, ethyl sebacate, geraniol extra, piperonal and vanillin fed at 1000 ppm for 27–28 wk (control group 10 males and 10 females).

(2) Anethole, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, Dolcourin, ethyl methylphenyl glycidate, ethyl pelargonate, ethyl sebacate, geraniol extra, isoeugenol, piperonal and vanillin fed at 10,000 ppm for 16 wk (control group 10 males and 10 females).
(3) Benzyl cinnamate, eugenol and thymol fed at 1000 and 10,000 ppm for 19 wk (control group 5 males and 5 females).
(4) Anethole, carvone and ethyl methylphenyl glycidate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).
(5) Allyl caproate and allyl cyclohexane propionate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).
(6) Ethyl vanillin and vanillin fed at 5000, 10,000 and 20,000 ppm for 2 yr (control group 12 males and 12 females).
(7) Ethyl vanillin and vanillin fed at 20,000 and 50,000 ppm for 1 yr (control group 5 males).
(8) Dihydrosafrole, isosafrole and safrole fed at 1000, 2500, 5000 and 10,000 ppm for 2 yr (control group 35 males and 35 females).

The animals were housed individually in wire cages and had access to food and water at all times. Most of the compounds were fed in the diet, but a few were administered by stomach tube. Fresh diets were made and distributed weekly except in the study with allyl isothiocyanate in which the diet was prepared and fed daily*. When the compound was administered by stomach tube, corn oil solutions of the flavouring were prepared. The concentration of the solution was adjusted for each level so that all rats received a constant volume of 1 ml of solution/kg daily. The control animals were given an equal volume of corn oil. The rat's weight, food intake and general condition were recorded every week. Haematological examinations were made at termination of the subacute studies, and at 3, 6, 12 and 22 months in the chronic experiments. These examinations included white cell counts, red cell counts, haemoglobins and haematocrits.

At the termination of the experiments the rats were sacrificed and exsanguinated. The tissues of all the rats were examined macroscopically at the time of sacrifice. The viscera were removed and the liver, kidneys, spleen, heart, and testes were weighed. These organs, the remaining abdominal and thoracic viscera, and one hind leg, for bone, bone marrow, and muscle, were preserved in 10% buffered formalin-saline solution for histopathological examination. For routine histopathology, sections were embedded in paraffin wax and stained with haematoxylin and eosin. Tissues from rats dying during the experiment were examined for gross changes and were preserved if autolysis was not advanced. Organs were not weighed but abnormalities and the suspected reason for death were noted.

Detailed microscopic examinations in the subacute studies were generally done on 6 or 8 rats, evenly divided by sex, from the high dose group and the control group. If changes attributable to the test compound were found in the high dose group, additional animals on lower dosage levels were examined as indicated. For animals from the long-term studies, more extensive histopathological study was done.

Dog studies. Pure-bred beagles were used in the studies with safrole and methyl salicylate. Mongrels and beagles were used in the coumarin, dihydrocoumarin, and 6-methylcoumarin studies. The dogs were wormed and immunized against distemper, rabies, and infectious

*Two other exceptions were the safrole rat study (Long *et al.* 1963) and the chronic rat feeding study of methyl salicylate (Webb & Hansen, 1963) in which the diets were prepared every other week and stored under refrigeration in sealed containers.

hepatitis before treatment was begun. They were housed in individual cages with food (commercial dry dog food) and water available at all times. The test compound was administered by capsule daily for 6 days a week. The animals were weighed weekly and the dosages were recalculated at this time. Haematological examinations were made three times prior to the start of treatment and at 2 wk, 1 month, 3 months, 6 months and 1 yr after treatment began and then at yearly intervals until termination of the study.

At the end of a study, survivors were killed with an overdose of an intravenous barbiturate and exsanguinated. The major organs were weighed. Sections of brain (four levels), pituitary, salivary gland, thyroid, parathyroid, thymus, lymph nodes, lung, heart, stomach, spleen, pancreas, kidney, adrenal, liver, gall bladder, small intestine (three levels), large intestine, urinary bladder, rib, skeletal muscle, and testis and prostate (or ovary and uterus) were preserved in 10% buffered formalin-saline for histological examination. In addition liver, kidney and adrenal sections and a femoral marrow plug were fixed in Zenker's solution. The sections were embedded in paraffin wax and stained with haematoxylin and eosin. Frozen sections of the liver and kidney were stained with Oil Red O for fat. A rib marrow smear was air-dried and stained with the Wright-Giemsa stain. Dogs found in a moribund condition during the experiment were sacrificed and necropsied. Dogs dying during the experiment were necropsied and tissue sections preserved if autolysis was not too advanced. All the tissues that were preserved were examined microscopically.

RESULTS

Table 1 shows the compounds tested; the dose levels; number, sex and species of test animals; duration; and effects. When the dose level is given in ppm, the test compound was mixed in the diet. When the dose is given in mg/kg, the test compound was administered daily by stomach tube to rats and (6 days/wk) by capsule to dogs. Only those effects believed to be due to the administration of the compound are listed. The designation "no effect" indicates that there was no effect on growth or haematology, and no macroscopic or microscopic change in the tissues. The designation "no macroscopic effect" indicates that there was no effect on growth or haematology, and no macroscopic change in the tissues, and that microscopic examination of the tissues was not performed. Pathological changes attributable to disease or age have not been included. For those compounds on which the toxicity studies have been reported previously the reference is given in Table 1 with the name of the compound.

In the evaluation of the data from the feeding studies with flavouring agents, the possibility of loss of the test compound from the experimental diet must be considered. Many of the flavouring agents used in these studies were quite volatile. Studies in our laboratory have shown that a decrease in the concentration of some of the flavourings occurred when the experimental diet was exposed in the open feed cups (Jones, Taylor, & Hagan, 1962). In order to determine the actual amount of the flavouring consumed by the animals, many of the experimental diets were analysed for the flavouring immediately after preparation and again after being exposed to laboratory conditions for 7 days. The flavourings on which analytical studies have been made and the percentage loss calculated over a 7-day period are presented in Table 2.

Table 1. *Summary of subacute and chronic toxicity of food flavourings and related compounds*

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Acetophenone (methyl 1-phenyl ketone)	10,000	Rat 10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Allyl butyrate (allyl butanoate)	90 mg/kg	do.	18 wk	Growth retardation in males. Liver: macroscopic—rough or granular surface, firm consistency, nutmeg appearance; microscopic—slight to moderate bile-duct proliferation and fibrosis with pseudolobule formation. Necrosis with polymorphonuclear infiltration and swollen, foamy liver cells in 2 of 8 rats examined
	50 mg/kg	do.	17 wk	Lung—slight to marked peribronchial lymphocytic infiltration[b]. Liver—no effect
Allyl caproate (allyl hexanoate)	2500	5M & 5F	1 yr	No effect
	1000	do.	28 wk	do.
	100 mg/kg	10M & 10F	18 wk	Liver: macroscopic — somewhat nodular and wrinkled with granular or rough surface; microscopic—slight to moderate bile-duct proliferation, some lobular architectural disarrangement, slight fibrosis, pigment in macrophages; necrotic foci in 2 of 8 rats examined
	65 mg/kg	do.	do.	Liver: microscopic—very slight bile-duct proliferation in 2 of 8 rats examined[c]
	15 mg/kg	do.	do.	No effect[c]
Allyl cyclohexane propionate (allyl 3-cyclohexylpropionate)	2500	5M & 5F	1 yr	No effect
	1000	do.	27–28 wk	do.
Allyl isothiocyanate[d]	10,000	do.	26 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.

[a]Unless otherwise stated.
[b]Controls had slight to marked peribronchial lymphocytic infiltration not as severe as with the 50 mg/kg/day dose.
[c]Microscopic examinations were made on the liver only.
[d]The diet was prepared daily because of the extreme volatility of this compound. A control group pair-fed with the 10,000 ppm group was included in this study in addition to the usual untreated control group.

[a]Unless otherwise stated.
[b]Controls had slight to marked peribronchial lymphocytic infiltration not as severe as with the 50 mg/kg/day dose.
[c]Microscopic examinations were made on the liver only.
[d]The diet was prepared daily because of the extreme volatility of t[...]und. A control group pair-fed with the 10,000 ppm group was included [...] hi[...] study in addition to the usual untreated control group.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Allyl isothiocyanate[d]	50 mg/kg	Rat 5M & 5F	20 days	Stomach: macroscopic—non-glandular part of the stomach was thickened, with lining occasionally roughened; microscopic—slight to moderate epithelial hyperplasia of the non-glandular part of the stomach, with acute to subacute ulcers from 2 to 6 1/2 mm across observed in all animals. Liver: microscopic—minor inflammatory foci
	20 mg/kg	do.	20 days	Stomach: macroscopic—same as seen in the 50 mg/kg animals; microscopic—the same type of ulceration as seen in the higher dose, occurring in 50% of the animals
Amyl butyrate	10,000	10M & 10F	16 wk	No effect
(pentyl butyrate)	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Amyl valerianate	10,000	do.	13 wk	No effect
(pentyl pentanoate)	1000	do.	do.	No macroscopic effect
Anethole (1-methoxy-4-propenylbenzene)	10,000	5M & 5F	15 wk	Liver: microscopic—slight hydropic changes of hepatic cells in males only
	2500	do.	1 yr	No effect
Anisaldehyde	10,000	do.	15 wk	No effect
(p-methoxybenzaldehyde)	1000	do.	27–28 wk	do.
Benzaldehyde	10,000	do.	16 wk	do.
	1000	do.	27–28 wk	do.
Benzyl cinnamate	10,000	do.	19 wk	do.
(cinnamein, benzyl β-phenylacrylate)	1000	do.	do.	No macroscopic effect
Calamus oil	10,000[e]	10M & 10F	18 wk	Dose-related growth depression, greater in males than in females. Increased mortality. Large amounts of clear fluid in the abdominal cavity. Liver: macroscopic—variable in appearance with some lobes reduced in size with rough surfaces and others smooth, abnormally rounded, and enlarged; microscopic—variation in cell size, capsular thickening, proliferation of bile duct epithelium, portal area fibrosis with haemosiderin deposition (females more severely affected than males). Heart—minimal to slight myocardial degeneration characterized by varying degrees of necrosis of muscle fibres, early fibrosis, and infiltration with mononuclear cells

[e]Young adult animals were used.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Calamus oil	5000	Rat[e] 10M & 10F	18 wk	Growth depression. Moderate amounts of clear fluid in the abdominal cavity. Liver—same changes as with 10,000 ppm but less severe. Heart—same changes as with 10,000 ppm but lower incidence
do.	2500	do.	do.	Growth depression. Liver—same changes as with 5000 and 10,000 ppm but less severe. Heart—same changes seen with the 10,000 ppm dose were seen in 1 of 6 rats examined
	1000	5M & 5F	do.	Growth depression
Carvone (*p*-mentha-6,8-dien-2-one)	10,000	do.	16 wk	Growth retardation, testicular atrophy
	2500	do.	1 yr.	No effect
	1000	do.	27–28 wk	do.
Cinnamic aldehyde (cinnamaldehyde)	10,000	10M & 10F	16 wk	Liver—slight hepatic cell swelling. Stomach—slight hyperkeratosis of squamous portion
	2500	do.	do.	No effect[f]
	1000	do.	do.	No macroscopic effect
Citral (3,7-dimethyl-2,7-octadienal)	10,000	do.	13 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	10,000[e]	3M & 3F	4 wk	Marked growth retardation, testicular atrophy. Liver: microscopic—slight to moderate damage consisting of dead and dying cells, decrease in oxyphilia and cytoplasm in the centrolobular cells, and proliferation of bile ducts
	10,000	10M & 10F	1–8 wk	Marked growth retardation. No animal survived beyond 8 wk. Liver: microscopic—slight to moderate damage of the type seen with the 10,000 ppm dose for 4 wk

[f]Microscopic examinations were made on the stomach only.
[g]Toxicity study will be presented in a separate report.
[h]Not acceptable for food use because of toxic effects observed in chronic feeding studies.

[f]Microscopic examinations were made on the stomach only.
[g]Toxicity study will be presented in a separate report.
[h]Not acceptable for food use because of toxic effects observe... chronic feeding studies.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[g,h] (2*H*-1-benzopyron-2 one)	1000	Rat 10M & 10F	14 wk	No effect
	1000	5M & 5F	28 wk	do.
	2500	6M & 6F	29 wk	Growth retardation. Liver: macroscopic—slight mottling in the males; microscopic—slight mid-zonal fatty change in both sexes but more marked in the males
	5000[i]	do.	2 yr	Growth retardation. Normal food utilization (measured for first year). Haemogram-decrease in haemoglobin. Liver: macroscopic—enlargement, distortion by poorly to well delineated masses, pale pin-point foci; microscopic—focal prolifera-tion of bile ducts of atypical appearance with associated fibrosis (cholangiofibrosis) situated in the masses described grossly and not clearly de-lineated from the surrounding hepatic paren-chyma; slight degree of fatty metamorphosis, very slight to slight architectural irregularity, very slight to slight variation in cell size, very slight proliferation of bile ducts of the normal type, minimal focal necrosis
	2500[i]	5M & 7F	2 yr	Liver—slight damage of the same type as seen with the 5000 ppm dose; cholangiofibrosis was not seen
	2500	6M & 6F	do.	Liver—minimal damage of the same type as seen with 5000 ppm dose; cholangiofibrosis was not seen
	1000[i]	do.	do.	No effect

[i]Corn oil added to diet at a level of 3% (w/w). Two control groups were included in the study. Corn oil was added to the diet of one control group; the diet of the other group was untreated.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[a,h] (2*H*-1-benzopyron-2-one)	100 mg/kg	Dog 1M & 1F	9–16 days	Male killed in extremis after 9 days. Female found dead on day 16. Marked emaciation, slight dehydration, and slight jaundice. Liver: macroscopic—moderate to marked yellow colour and moderate "nutmeg" appearance; microscopic—marked disorganization of the lobular pattern, moderate increase in size of liver cells (with variation ranging from slight atrophy to a great increase), slight to moderate vacuolation, moderate to large amount of diffusely distributed fat, slight to moderate focal necrosis, fibrosis ranging from slight (centrolibular) to moderate to marked (portal) and very slight to moderate bile-duct proliferation. Spleen—macroscopic moderately pale. Gastro-intestinal tract—intestinal contents thick, dark, and tarry. Bone marrow—thin and fatty. Gall bladder—moderately distended
	50 mg/kg	2M & 1F	35–277 days	Emaciation; slight to moderate jaundice in the two female dogs. One death at 35 days; remaining dogs sacrificed at intervals between 45 and 277 days. Liver: macroscopic—moderately yellow colour with "nutmeg" appearance; microscopic—the same changes as with the 100 mg/kg dose except that the liver cell vacuolation was greater. Spleen—large amount of haemosiderin. Bone marrow—pale
	25 mg/kg	do.	133–330 days	Moderate emaciation and slight jaundice in one female. Weight gain in remaining dogs. Liver—slightly pale in two dogs, dark red in a third and yellow, markedly "nutmeg" in the fourth; microscopic—liver changes like those seen on the 50 mg/kg dose to the same degree in one dog and less severe in the other three. Spleen—moderate haemosiderosis. Gall bladder—moderately distended in two animals
	10 mg/kg	2M & 2F	297–350 days	No definite effect
Dihydroanethole (1-methoxy-4-propylbenzene)	10,000	Rat 10M & 10F	19 wk	Bone—slight osteoporosis of the tibia and femur (6/10)
	2500	do.	do.	Bone—slight to very slight osteoporosis (6/10)
	1000	do.	do.	Bone—very slight osteoporosis[j] (4/10)

[j]In the control group slight osteoporosis was seen in one of 10 animals.

Dihydroanethole (1-methoxy-4-propylbenzene)	10,000	[illegible] & 10F	[illegible]	Bone—[illegible] (6/10)
	2500	do.	do.	Bone—slight to very slight osteoporosis (6/10)
	1000	do.	do.	Bone—very slight osteoporosis (4/10)

In the control group slight osteoporosis was seen in one of 10 animals.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydroanethole (1-methoxy-4-propylbenzene)	Maximal dose 5000 mg/kg	Rat 20M	32 days	Initial dose 2000 mg/kg increased gradually to 5000 mg/kg. Seven of the animals survived the full 32 days of treatment and 16 lived long enough to receive the maximal daily dose of 5000 mg/kg Stomach: macroscopic—mucosa of the fore-stomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—moderate degree of osteoporosis
Dihydrocoumarin (1,2-benzohydropyrone)	10,000	10M & 10F	14 wk	No effect
	1000	do.	do.	do.
	150 mg/kg	Dog 1F	2 yr	do.
	50 mg/kg	1M & 1F	do.	do.
Dihydrosafrole[h] (1,2-methylenedioxy-4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	do.	Growth retardation in both sexes. Oesophagus—benign epidermoid papillomas and malignant papillary epidermoid carcinomas; 75% of the rats showed oesophageal tumours, 50% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as that seen in the safrole-treated rats (see safrole rat—2-yr feeding study). Spleen—moderate follicular atrophy
	5000	25M & 25F	do.	Growth retardation in both sexes. Oesophagus—74% of the rats showed oesophageal tumours, 32% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen in the safrole-treated rats. Spleen—moderate follicular atrophy
	2500	10M & 10F	do.	Growth retardation in the females. Oesophagus—20% of the rats showed oesophageal tumours, 5% of which were malignant. Liver: macroscopic—enlargement; microscopic—slight liver damage of the same type as seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydrosafrole[h] (1,2-methylenedioxy 4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.* 1965)	1000	Rat 10M & 10F	2 yr.	Growth retardation in the females. Liver: macroscopic—slight enlargement; microscopic—slight liver damage of the same type as that seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis
Dimethylbenzyl carbinol	10,000	5M & 5F	16 wk	No effect
(1,1-dimethyl-2-phenylethanol)	1000	10M & 10F	28 wk	do.
Dolcourin[k]	10,000	5M & 5F	16 wk	do.
Ethyl caprylate	10,000	10M & 10F	17 wk	do.
(ethyl penanoate)	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl formate	10,000	do.	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl methylphenyl glycidate (ethyl ester of 2,3-epoxy-3-methyl-3-	10,000	5M & 5F	16 wk	Growth retardation most obvious in males Testes—marked atrophy
phenylpropionic acid)	2500	do.	1 yr	No effect
Ethyl oenanthate	10,000	10M & 10F	13 wk	do.
(ethyl heptanoate)	1000	do.	do.	No macroscopic effect
Ethyl oxyhydrate[l]	10,000	do.	17–18 wk	Liver: microscopic—very slight swelling of hepatic cells in males
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl pelargonate (ethyl nonanoate)	10,000	5M & 5F	16 wk	No effect
Ethyl sebacate	10,000	do.	17–18 wk	do.
	1000	do.	27–29 wk	do.
Ethyl vanillin	50,000[m]	5M	1 yr	do.
(3-ethoxy-4-hydroxybenzaldehyde)	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
Eugenol	10,000	10M & 10F	19 wk	do.
(1-hydroxy-2-methoxy-4-allylbenzene)	1000	do.	do.	No macroscopic effect

[k]Dolcourin is a trade mark name for a substance supplied by Dodge and Olcott, Inc. as a coumarin substitute.
[l]Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.
[m]3% (w/w) corn oil added to control and test diets as a binder to reduce evaporation of the flavouring.
[n]3% (w/w) propylene glycol added to control and test diets as a binder to reduce evaporation of the flavourings.

[l]Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used [illegible]
Labs., Inc., N.Y.
[m]3% (w/w) corn oil added to control and test diets as a b[illegible]duce evaporation of the flavouring.
[n]3% (w/w) propylene glycol added to control and test diet[illegible] binder to reduce evaporation of the flavourings.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	Maximal dose 4000 mg/kg	Rat 20M	34 days	Initial dose 1400 mg/kg increased gradually to 4000 mg/kg. Eight of the animals survived the full 34 days of treatment and 15 lived long enough to receive the maximal dose of 4000 mg/kg. Stomach: macroscopic—mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—small degree of osteoporosis
Eugenyl acetate (1-acetoxy-2-methoxy-4-allylbenzene)	10,000	10M & 10F	19 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Geraniol extra (3,7-dimethyl-2,6-octadienol) (3,7-dimethyl-1,6-octadienol)	10,000	5M & 5F	16 wk	No effect
	1000	do.	27–28 wk	do.
Geranyl acetate	10,000	10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ionone standard [60% α-ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one and 40% β-ionone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one]	10,000	do.	do.	Liver: microscopic—slight to moderate swelling of parenchymal cells
	2500	do.	do.	Liver: microscopic—slight amount of swelling of parenchymal cells[e]
	1000	do.	do.	Liver: microscopic—very slight amount of swelling of the parenchymal cells[e]
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)	10,000	5M & 5F	16 wk	No effect
Isosafrole[h]	10,000	10M & 10F	11 wk	Growth retardation in both sexes. No rats on this dose survived beyond 11 wk of treatment. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole
(Hagan *et al.* 1965)	5000	25M & 25F	2 yr	Growth retardation in both sexes. Testes—increased number of interstitial cell tumours. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole. Five rats had primary hepatic tumours (two of these had hepatic cell adenomas and three had hepatic cell carcinomas). Kidney—increased incidence of chronic nephritis. Thyroid—slight hyperplasia

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Isosafrole[b] (Hagan *et al.* 1965)	2500	Rat 10M & 10F	2 yr	Slight growth retardation in females. Liver: macroscopic—slightly enlarged; microscopic—slight damage of the same type as seen with safrole, no primary hepatic tumours. Thyroid—slight hyperplasia
	1000	do.	do.	Slight growth retardation in females. Liver: microscopic—slight damage of the same type as seen with safrole
Linalyl cinnamate	10,000	10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Linalyl isobutyrate	10,000	do.	18 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Methyl anthranilate (*o*-aminobenzoic acid, methyl ester)	10,000	do.	13 wk	No effect
	1000	do.	do.	No macroscopic effect
6-Methylcoumarin (6-methyl-2*H*-1-benzopyran-2-one)	15,000	25M & 25F	2 yr	Depression of growth, severe in males and moderate in females, and paralleled by decrease in food intake. Liver: microscopic—slight fatty metamorphosis, and very slight bile-duct proliferation and focal telangiectasia. Testes—moderate atrophy, probably due to growth retardation
	7500	do.	do.	Depression of growth in males
	3500	do.	do.	No effect
	1000	do.	do.	do.
	500	do.	do.	do.
	10,000	10M & 10F	14 wk	do.
	1000	do.	do.	do.
	150 mg/kg	Dog 1M	39 days	Sacrificed because of weakness, emaciation, dehydration. Liver: microscopic—moderate to severe hepatitis. Skeletal muscle—slight to moderate atrophy
	50 mg/kg	1M & 1F	2 yr	No effect

severe hepatitis. Skeletal muscle—slight to moderate atrophy

50 mg/kg 2 yr No effect

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Methyl salicylate (o-hydroxybenzoic acid, methyl ester)	20,000	Rat 24M & 26F	49 wk	Growth retardation, rough coats. All dead by wk 49. Bone: microscopic—excess of cancellous tissue
(Webb & Hansen, 1963)	10,000	25M & 25F	2 yr	Growth retardation, rough coats. Bone: microscopic—slight excess of cancellous tissue
	5000	do.	do.	Bone: microscopic—slight excess of cancellous tissue
	1000	do.	do.	No effect
	10,000	10M & 10F	17 wk	Growth retardation
	1000	do.	do.	No macroscopic effect
	1200 mg/kg	Dog 1M & 1F	3 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	800 mg/kg	1M	29 days	Sacrificed, weight loss. Liver: microscopic—trace of fat
	800 mg/kg	1F	4 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	500 mg/kg	1M & 1F	8–9 days	Sacrificed—No effect
	250 mg/kg	do.	51–52 days	do.
	100 mg/kg	do.	52–53 days	do.
	50 mg/kg	do.	53–59 days	do.
	350 mg/kg	2M & 1F	2 yr	Weight loss. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	150 mg/kg	2M & 2F	do.	Growth retardation. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	50 mg/kg	do.	do.	No effect
Phenylethyl phenylacetate	10,000	Rat 10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Piperonal (3,4-methylenedioxybenzaldehyde)	10,000	5M & 5F	15 wk	No effect
	1000	do.	27–28 wk	do.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[h] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	62 wk	Growth retardation in both sexes. All rats dead by wk 62. Testes—atrophy. Stomach—atrophy and atypical regeneration of the mucosa glands with associated fibrosis and hyalinization of the surrounding stroma most severe at the base. Liver: macroscopic—enlarged (2 to 3 times the normal size), mottled and irregularly nodular, with single or multiple tumour masses; microscopic—hepatic cell enlargement, which was usually focal and resulted in the formation of nodules. The enlargement was chiefly of the cytoplasm, which appeared normal in some cells but showed fatty metamorphosis or peripheral migration of the basophilic cytoplasmic granules in other cells. The nodules tended to progress in one of 3 ways (sometimes all 3 were seen in the same liver): (1) cystic necrosis; (2) cirrhosis; and (3) adenomatoid hyperplasia, leading to the formation of both benign and malignant tumours (hepatic cell adenoma, hepatic cell carcinoma, hepatocholangioma, hepatocholangiocarcinoma)
	5000	25M & 25F	2 yr	Growth retardation in both sexes. Increased mortality in the males. Liver—changes of the same type and severity as seen with the 10,000 ppm dose except for the occurrence of macroscopic cysts and a higher incidence of tumours in the lower dose, statistically-significant increase in malignant primary hepatic tumours. Stomach—same type of changes as with 10,000 ppm dose. Kidney—slight increase of chronic nephritis in the females. Thyroid—mild hyperplasia
	2500	10M & 10F	do.	Growth retardation in both sexes. Liver—moderate liver damage of the same type as seen with the 10,000 ppm dose with the exception of cirrhosis. Kidney—moderate increase of incidence of chronic nephritis.

	2500	[illegible]F	do.	Growth retardation in both sexes. Liver—[illegible] liver damage of the same type as see[illegible] 10,000 ppm dose with the exception of cirrhosis. Kidney—moderate increase of incidence of chronic nephritis.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[h] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	1000	Rat 10M & 10F	2 yr	Growth retardation in the females. Liver—slight liver damage of the type seen with the 10,000 ppm dose, except that malignant tumours and cirrhosis were not seen with this dose. Kidney—moderate increase in chronic nephritis in the males.
(Long *et al.* 1963)	5000	25M & 25F	do.	Growth retardation in both sexes. Haemograms—mild anaemia, slight leucocytosis. Liver—marked liver changes (including malignant tumours but only rare cirrhosis) of the type seen with the 10,000 ppm dose. Statistically-significant increase in benign and malignant primary liver tumours.
	1000	25M & 25F	do.	Liver—slight to moderate damage of the same type seen with the 10,000 ppm dose except that malignant tumours and cirrhosis were not seen
	500	do.	do.	Liver—slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	100	do.	do.	Liver—very slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	80 mg/kg	Dog 2M & 2F	26–39 days	One dog died; the remaining dogs were sacrificed in a moribund condition. Marked weight loss. Skeletal muscle—moderate to severe atrophy in 2 of 4 dogs. Liver: macroscopic—slight enlargement in 2 of 4 dogs; microscopic—heavy infiltration of fat, occasional bile-duct proliferation and inflammatory-cell infiltration, slight to moderate streaking with necrobiosis and liver cell atrophy
	40 mg/kg	do.	91–116 days	Emaciation, general weakness. Liver: macroscopic—slight enlargement in 3 of 4 dogs; microscopic—slight fat infiltration, moderate hepatic cell atrophy and necrobiosis as at 80 mg/kg

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[h] (1,2-methylenedioxy-4-allylbenzene (Long *et al.* 1963)	20 mg/kg	Dog 2M & 2F	6 yr	Liver: macroscopic—slight to moderate enlargement, nodular surface; microscopic—mild post-necrotic cirrhosis characterized by focal or generalized nodules, consisting of enlarged hepatic cells with slight focal fatty vacuolization, separated by bands of atrophied hepatic cells and collagen; slight lymphocytic, Kupffer cell and bile-duct proliferation; minimal focal necrosis. Thyroid—hyperplasia
	5 mg/kg	do.	do.	Liver: microscopic—minimal focal necrosis, bile-duct proliferation, fatty metamorphosis, hepatic cell atrophy and leucocytic infiltration
Terpinyl acetate (*p*-menth-1-en-8-ol ester of acetic acid)	10,000	Rat 10M & 10F	20 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Thymol (*p*-cymen-3-ol; hydroxy-*p*-cymene)	10,000	5M & 5F	19 wk	No effect
	1000	do.	do.	No macroscopic effect
γ-Valerolactone (4-hydroxypentanoic acid lactone)	10,000	10M & 10F	13 wk	No effect
	5000	do.	do.	do.
	2500	11M & 9F	do.	do.
	1000	10M & 10F	do.	do.
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	50,000[m]	5M	1 yr	do.
	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
	10,000	5M & 5F	16 wk	do.
	1000	do.	27–28 wk	do.

Table 2. *Loss of food flavourings from laboratory animal diets during a 7-day period*

Flavouring	Percentage loss*	Flavouring	Percentage loss*
Acetophenone	31	Eugenyl acetate†	6
Allylcaproate	31	Geranyl acetate†	4
Amyl butyrate†	35	Ionone†	1
Anethole	6	Isosafrole	5
Calamus oil	0	Linalyl cinnamate	4
Cinnamic aldehyde	14	Linalyl isobutyrate	7
Citral	58	Methyl salicylate†	3 & 5§
Dihydroanethole†	16	Phenylethyl phenylacetate	0
Dihydrocoumarin	—‡	Safrole	12
Dihydrosafrole	6	Terpinyl acetate	7
Ethyl caprylate†	33		

*100 − (100 × day 7 recovery/day 0 recovery).
†Reported by Jones *et al.* (1962). Recovery data on the remaining compounds were supplied by W. I. Jones.
‡Dihydrocoumarin was partially converted to melilotic acid when mixed with the laboratory diet; the test animals ingested a mixture of the two compounds.
§The two values for percentage loss indicate two different analytical procedures.

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Substances d'assaisonnement et composés de structure connexe. II. Toxicité subaiguë et chronique

Résumé—On a étudié sur des rats la toxicité subaiguë et chronique de 48 substances d'assaisonnement. On a également étudié sur des chiens la toxicité de cinq d'entre elles (coumarine, 6-méthylcoumarine, dihydrocoumarine, salicylate de méthyle et safrol). On a présenté un résumé de ces études. On a déterminé aussi la deperdition de 21 substances d'assaisonnement en 7 jours, dans des régimes pour animaux de laboratoire.

Lebensmittel-Geschmacksstoffe und Verbindungen verwandter Struktur. II. Subakute und chronische Toxizität

Zusammenfassung—Die subakute und bzw. oder chronische Toxizität von 48 Lebensmittel-Geschmackszusätzen wurde an Ratten untersucht. Die Toxizität von fünf dieser Geschmacksstoffe (Cumarin, 6-Methylcumarin, Dihydrocumarin, Methylsalicylat und Safrol) wurde auch an Hunden untersucht. Eine Zusammenfassung der Versuchsergebnisse wird vorgelegt. Der Verlust von 21 Geschmacksstoffen aus dem Futter von Laboratoriumstieren während einer 7-Tage-Periode wurde ebenfalls bestimmt.

#64

TOXICOLOGY AND APPLIED PHARMACOLOGY 7, 18-24 (1965)

Toxic Properties of Compounds Related to Safrole

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Dihydrosafrole (1,2-methylenedioxy-4-propylbenzene) and isosafrole (1,2-methylenedioxy-4-propenylbenzene) are chemically related to safrole (4-allyl-1,2-methylenedioxybenzene); allyl heptylate, a pineapple flavor, to safrole through a common allyl moiety. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene) is the principal ingredient of oil of cloves; piperonal (3,4-methylenedioxybenzaldehyde) contributes to vanilla and

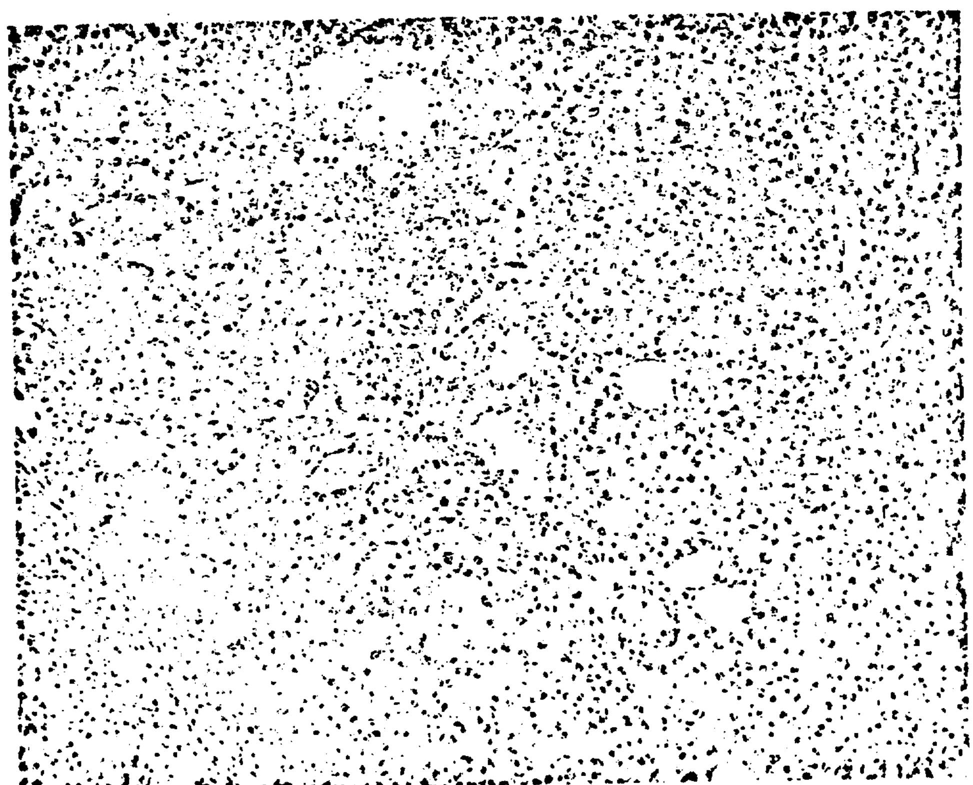

FIG. 1. Liver of rat given 5000 ppm safrole in the diet for 89 weeks. The grade 1 trabecular hepatic cell carcinoma at the right, with its sinusoidal telangiectasia and trabeculae two or more cells in thickness composed of enlarged cells with hypertrophied nuclei, is clearly separated by a thin fibrous band from the nonneoplastic fragment of liver at the left. Hematoxylin and eosin. Magnification: × 125.

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s Related to Safrole

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nzene) and isosafrole (1,2-methyl-
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zene) is the principal ingredient of
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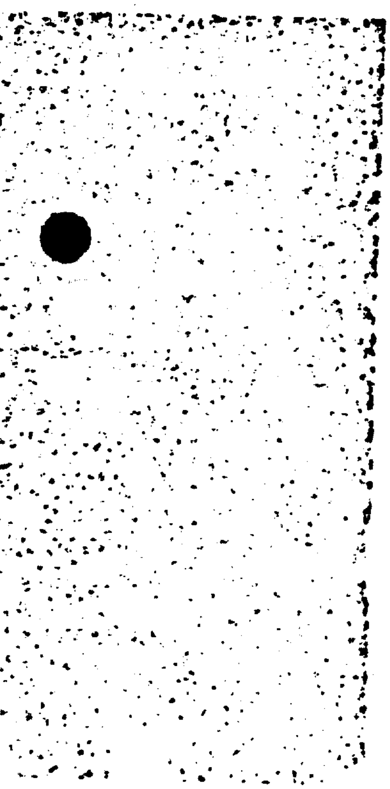

for 89 weeks. The grade 1 trabecular
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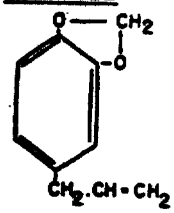
lle, Indiana.

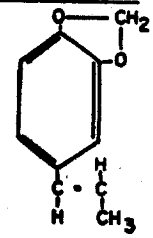
cherry flavors. Allylbenzene and methylenedioxybenzene are related chemicals. Safrole is reported as producing benign and malignant liver tumors (Fig. 1) when fed to rats for chronic periods (Long *et al.*, 1963).

Because of the use of some as flavors, and of chemical relationships, a study of their toxic effects was considered desirable.

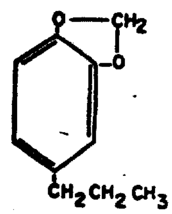
SAFROLE



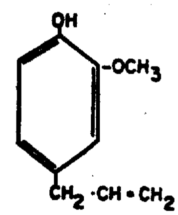
ISOSAFROLE



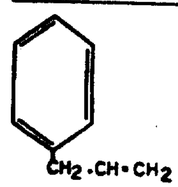
DIHYDROSAFROLE



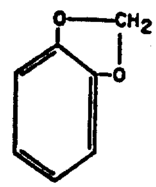
EUGENOL



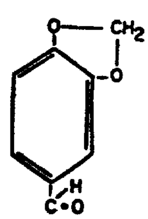
ALLYL BENZENE



METHYLENEDIOXYBENZENE



PIPERONAL



ALLYL HEPTYLATE

$CH_3\,(CH_2)_4\,COOCH_2\cdot CH{=}CH_2$

METHODS

Acute Toxicity

Oral LD_{50}s were obtained by administering undiluted or corn oil concentrations of the materials by stomach tube to young adult rats, mice, and guinea pigs. Food was withdrawn 18 hours prior to dosing rats and guinea pigs; mice were treated on full stomachs.

Subacute Toxicity

Groups of young adult Osborne-Mendel rats of both sexes received increasing doses of dihydrosafrole and isosafrole by oral intubation for periods ranging up to 105 days. Safrole was administered as a comparative measure since it produced liver changes previously by subacute as well as chronic administration.

Increasing doses of eugenol ranging from 1400 to 4000 mg/kg were administered similarly to rats for 34 days. Six groups of Swiss mice were treated with daily doses of 250 and 500 mg/kg daily of allylbenzene, methylenedioxybenzene, and safrole for 60 days. Allyl heptylate was fed to weanling Osborne-Mendel rats of both sexes at dietary levels of 10,000, 2500, 1000, and 0 ppm for 18 weeks, and piperonal at 10,000 and 1000 ppm for 16 and 28 weeks, respectively.

Chronic Toxicity

I. Dihydrosafrole, safrole, and isosafrole were fed for 2 years to groups of 50 weanling rats (25 males; 25 females) at 5000 ppm. Additional groups of 20 rats (10 males; 10 females) were fed 10,000, 2500, 1000, and 0 ppm for the same period.

II. Four groups of six beagle dogs, evenly distributed by sex, were started on allyl heptylate at doses of 75, 25, 5, and 0 mg/kg/day administered by capsule.

RESULTS

Acute and Subacute Oral Toxicity

Toxicologic findings are summarized in Tables 1 and 2.

Daily administration of eugenol by stomach tube resulted in a few deaths at 2000 mg/kg, but mortality was augmented as dosage was increased to 4000 mg/kg. This is understandable since for rats the acute LD_{50} is 2680 mg/kg (Table 1).

Methylenedioxybenzene was somewhat more toxic in mice than safrole at daily doses of 500 mg/kg; percentage mortalities were 81 and 65, respectively. At 250

TABLE 1
ACUTE ORAL TOXICITY

Compound	Animal	Sex	LD_{50} (mg/kg)[a]	Slope function
Allylbenzene	Rat	M&F	5540 (4620–6650)	1.5 (1.2–2.0)
	Mouse		2900 (2500–3360)	1.4 (1.1–1.7)
Allyl heptylate	Rat		500 (392–638)	1.7 (1.2–2.2)
	Mouse		630 (514–772)	1.5 (1.2–1.8)
	Guinea pig		444 (363–541)	1.6 (1.2–2.3)
Dihydrosafrole	Rat		2260 (1840–2780)	1.7 (1.4–2.0)
	Mouse		3700 (3010–4550)	1.2 (1.1–1.4)
Eugenol	Rat		2680 (2420–2970)	1.2 (1.1–1.4)
	Mouse		3000 (2400–3750)	1.8 (1.3–2.3)
	Guinea pig		2130 (1860–2430)	1.3 (1.2–1.5)
Isosafrole	Rat		1340 (1140–1590)	1.4 (1.2–1.7)
	Mouse		2470 (2010–3040)	1.7 (1.3–2.2)
Methylenedioxybenzene	Rat		580 (487–690)	1.4 (1.2–1.7)
	Mouse		1220 (976–1520)	1.4 (1.2–1.7)
Piperonal	Rat		2700 (2350–3100)	1.5 (1.1–2.0)
Safrole	Rat		1950 (1760–2160)	1.3 (0.6–3.0)
	Mouse		2350 (2010–2750)	1.4 (1.1–1.6)

[a] Litchfield and Wilcoxon (1949).

TABLE 2

LETHAL EFFECTS OF RATS RECEIVING INCREASING DOSES OF SAFROLE AND RELATED COMPOUNDS

Compound[a]	Concentration in corn oil (%)	Dose (mg/kg/day)	Mortality ratio	Duration (days)
Isosafrole	25	250	2/10	34
		500	8/10	41
Dihydrosafrole	25	250	0/10	34
		500	0/10	46
		750	3/10	26
Safrole	25	250	0/10	34
		500	1/10	46
		750	9/10	19
Control		Corn oil	0/10	106

[a] Ten rats per compound and control.

mg/kg/daily mortalities of 73 and 38% resulted, respectively. Allylbenzene in the same dosage produced 29 and 21% mortalities. Methylenedioxybenzene produced both stimulation and depression, whereas allylbenzene and safrole produced only depression.

Allyl heptylate produced severe growth depression in both male and female rats at 10,000 ppm—males to a lesser extent at lower levels consistent with dose. Poor food efficiency (requiring more food per gram of gain) resulted at 10,000 ppm, but not at 2500 and 1000.

No adverse effects occurred in the rats fed piperonal.

Chronic Oral Toxicity

Rats fed isosafrole in a projected 2-year study survived less than 10 weeks at the 10,000 ppm level. At the same level of feeding, 35% of the dihydrosafrole animals survived 50 weeks and 20%, 75 weeks. Conversely, at the 0.5% level, mortality at 75 and 100 weeks was greater with dihydrosafrole (34 and 90%) than isosafrole (10 and 56%).

All dogs fed allyl heptylate in daily doses of 75 mg/kg succumbed in from 3 to 7 months, while dogs being fed 25 and 5 mg/kg/day are currently surviving at 18 months on experiment without gross effects.

Pathology

Subacute studies. Rats given isosafrole, safrole, and dihydrosafrole subacutely by stomach tube exhibited, on macroscopic examination, liver enlargement and adrenal enlargement with yellow discoloration. This was most marked in rats given safrole and dihydrosafrole. Microscopic examination revealed an average moderate degree of pathology in the liver in all three groups. The most striking change was hepatic cell enlargement, chiefly due to an increase in cytoplasm and occurring in all test groups. Variation in hepatic cell size ranged from slight with safrole and isosafrole to moderate with dihydrosafrole. Other consistent changes were slight degrees of focal fatty metamorphosis, bile duct proliferation, and architectural irregularity. Safrole and isosafrole also produced slight focal necrosis and fibrosis. Microscopically, the adrenals of rats given safrole and dihydrosafrole showed an increase in lipid in the cytoplasm of the cortex accounting for the hypertrophy and deep yellow color noted grossly.

Eugenol produced slight liver enlargement and slight adrenal enlargement with marked yellowish discoloration. The liver enlargement noted grossly was found on microscopic examination to be due to the slight degree of liver cell enlargement. The forestomach showed a moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration.

Safrole produced liver changes in mice typical of those observed in rats in contrast to negative and minimal liver effects observed with allylbenzene and methylenedioxybenzene, respectively.

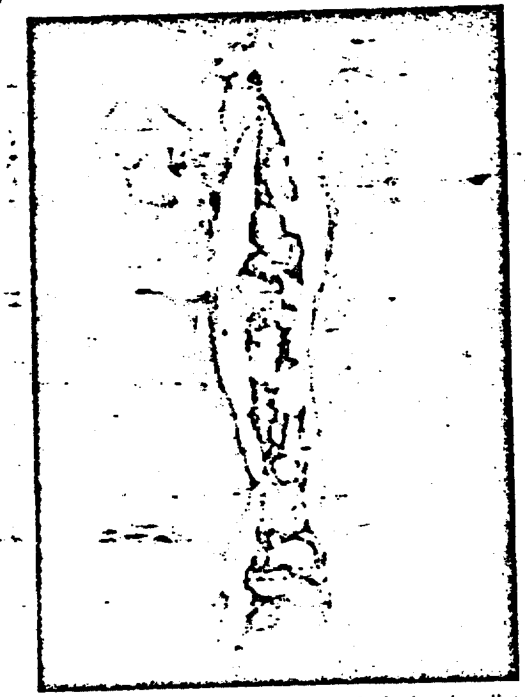


FIG. 2. Esophagus of rat given 5000 ppm dihydrosafrole in the diet for 104 weeks. Multiple growths of white, friable, granular, papillary material project from the mucosa into the lumen both as (1) discrete polyps 3–4 mm in diameter arising from thin stalks up to 5 mm in length, and (2) coalescent, sessile tumors of similar elevation. The tumor masses plus entrapped food had resulted in partial obstruction with ensuing dilatation of the lumen. The white masses in the lungs represent pneumonia, not metastatic carcinoma. Hematoxylin and eosin. Magnification: × 1.7.

Allyl heptylate produced in rats gross liver enlargement at 10,000, 2500, and 1000 ppm; enlarged kidneys in both males and females and enlarged hearts in males both at the highest level; and enlarged testes at the highest and next highest levels. Microscopic changes consisted of hydropic degeneration of the liver cells in the periportal areas ranging from moderate at the highest level to lesser degrees at lower levels, and corresponded to dosage. Extent of new bile duct formation correlated with degree of hydropic degeneration. Hepatic cell enlargement was noted in some groups.

Effects of feeding safrole, dihydrosafrole, and isosafrole for chronic periods. Dihydrosafrole induces the formation of esophageal tumors, both benign epidermoid papillomas and malignant papillary epidermoid carcinomas (Fig. 2 and 3), in the 10,000 and 5000 ppm groups. Seventy-five per cent of the rats fed the high level showed incidence of tumor formation, 50% of tumors being malignant, and at 5000 ppm a total incidence of 74% with 32% malignant. The controls were free of tumors. Because of this and the extremely low spontaneous incidence of these tumors in this rodent in general, dihydrosafrole may be considered a definite esophageal carcinogen for the rat.

FIG. 3. A higher power of an esophageal tumor similar to one of those shown in Fig. 2. This grade 1 papillary epidermoid carcinoma consists of a stalk extending into the lumen from the mucosa and covered by projecting papillae of acanthotic, heavily keratinized stratified squamous epithelium which is invading the underlying lamina propria. Hematoxylin and eosin. Magnification: $\times$ 19.

All three compounds produced in the liver the same general type of pathology seen in the first experiment with safrole. The major changes included hepatic cell enlargement, which was usually focal and resulted in the formation of nodules; adenomatoid hyperplasia; cystic necrosis; fatty metamorphosis; and bile duct proliferation. However, in contrast to safrole, which produced severe damage, overall pathology was slight at all levels of treatment with the other two compounds. Although a few tumors were found in the livers of the animals fed isosafrole and dihydrosafrole, there were not enough to be of statistical significance. Hence, unlike safrole, which is a definite hepatic carcinogen, neither isosafrole nor dihydrosafrole should be considered tumorigenic for the liver.

Dihydrosafrole produced a moderate increase in chronic nephritis at 2500 and 1000 ppm; the apparent lack of effect at the two upper levels of dosage probably was the result of the poor survival.

The administration of allyl heptylate at 75 mg/kg/day in dogs depressed growth, and produced liver and stomach changes consisting of mottled appearance with rough surfaces in the former and hemorrhagic mucosae in the latter. Changes observed less constantly were small gray or red cysts of clear aspect in the urinary bladders, and marked congestion in lungs, digestive tracts, kidneys, spleens, and lymph nodes. Some organs exhibited terminal hemorrhages.

On microscopic examination all livers showed a slight to moderate fibrosis of portal areas, tending to surround hepatic cell lobules of irregular size and shape and associated with slight to moderate proliferation of bile duct epithelium. Slight increases of fat occurred in all but one instance. The stomach presented diffuse hemorrhage and some necrosis of the mucosae together with instances of focal submucosal hemorrhages.

SUMMARY

Dihydrosafrole on chronic feeding produced benign and malignant esophageal tumors. Safrole is a hepatic carcinogen. Liver changes produced by isosafrole, safrole, and dihydrosafrole were of the same general type and included hepatic cell enlargement, which was usually focal and resulted in the formation of nodules; adenomatoid hyperplasia; cystic necrosis; fatty metamorphosis; and bile duct proliferation. The magnitude of liver changes was much greater with safrole than with the other two compounds. Safrole produced liver changes in mice. Similar treatment with the allylbenzene and methylenedioxybenzene moieties of safrole produced changes only with methylenedioxybenzene and were less than that observed with the parent compound. This was confirmed in rats by Taylor *et al.* (1964).

All compounds studied produced liver alterations with the exception of allylbenzene and piperonal.

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being CFP, A, P. There were also differences in latencies between the two general categories. Table 1 shows that latencies of cells in a preferentially responsive class were in all three instances less than latencies of equally responsive cells firing to the same modality of stimulation.

These data are all consistent with the interpretation that an important portion of a given population of cells in association cortex can be expected to demonstrate differential responsiveness to converging sensory input. Another portion would show equal convergence. This fact is supported by the results given in Table 1 and also by instances of occlusive interactions observed when firing probabilities were sufficiently high for such tests to be made.

Simultaneous pairing of two stimuli yielded unequivocal data on cellular integrative mechanisms. Sixty cells were tested with pairings of supra- and/or sub-threshold stimuli. Fifteen cells showed summation of input, seven facilitation (one stimulus of the pair not producing any cell discharge alone), and five inhibition. Summation, as illustrated in Fig. 3, could be seen in some cells only with pairing of all three inputs. With the cell illustrated a distinct latency decrease occurred, plus the appearance of additional cell spikes.

These results show that with a simple flexible microelectrode reliable extracellular recordings can be obtained from cortical neurones for at least many hours. The data also indicate that the anterior marginal gyrus of the cat contains neurones unequally responsive to different modalities of sensory input and it has cellular discharge characteristics reflecting summation, facilitation and inhibition.

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Inhibition of β-D-Glucosiduronic Acid Conjugation by Eugenol

IT has been claimed that eugenol, 4-oxy-3-methoxy-1-allylbenzoic acid, has mucinogenic activity and that it has a beneficial effect on gastric ulcers[1,2]. The gastric wall contains various uronic acid containing mucopolysaccharides, the formation of which is possibly dependent on a proper coupling capacity of β-D-glucosiduronic acid (UDPGA). Evidence has been obtained which indicates that the amount and type of mucopolysaccharides in tissues probably have some bearing on the mechanism of ulcer formation[3]. This investigation was undertaken in order to determine whether eugenol has any effect on the glucuronation of an exogenic substance. The effect on the whole conjugation capacity of the tissue, as well as on the UDPGA transferase enzyme, was also considered in the investigation.

Wistar rats and guinea-pigs were used as test subjects. The whole conjugation was followed using a modification of the method developed by Levvy and Storey[5,6]. The UDPGA transferase activity was determined by the method of Bogell and Leloir[7]. This method requires washed microsomes, UDPGA (Sigma) and *p*-nitrophenol. Eugenol was dissolved in 0·2 per cent 'Triton X-100' solution. The oxygen consumption was measured in a Warburg apparatus.

The effect of eugenol on the formation of *o*-aminophenol glucuronide by liver slices is shown in Fig. 1. It can be seen that a 50 per cent inhibition is obtained with a eugenol concentration of 2 μg/ml. in the incubation solution. The same result was obtained using 50 and 100 μg aminophenol. At a concentration of 200 μg/ml. or above, eugenol constantly caused an activity less than 5 per cent of that of the controls; 250 μg resulted in some cloudiness. At a concentration of 100 μg/ml. the oxygen consumption was identical to that of both the controls and also to the controls without the tissue specimens.

Several additional experiments were carried out in order to elucidate the nature of the inhibitory effect of eugenol. When eugenol (100 μg) was added after the tissue had been incubated for 1 h, a 70 per cent reduction in glucuronide synthesis was produced as compared with the control.

Eugenol, when added just before deproteinization and at subsequent stages, had no effect. This eliminates the possibility of there having been any interference in the analytical procedure.

In the *in vivo* experiments, 3 ml. of 5 per cent eugenol was given to the animals and this virtually resulted in a complete inhibition in rinsed stomach, and about a 75 per cent inhibition in duodenal slices. The results with liver were just above normal. No effect could be detected after 24 h, but it was still complete 6 h after the eugenol had been administered; the effect was variable after 12 h. Histological studies of the stomach revealed desquamation of the surface epithelium and punctate haemorrhages in the pylorus and glandular stomach. Cellular exudate was present on the surface; this was visible to the naked eye. No attempt was made to find the least effective dose *in vivo*. When tissue slices were placed in a solution of 0·025 per cent eugenol in Ringer's solution for 2 min before they were rinsed and transferred to the Warburg vessel, the synthesis dropped to less than 25 per cent of the controls. The effect was the same for stomach, duodenum and liver. No attempt was made,

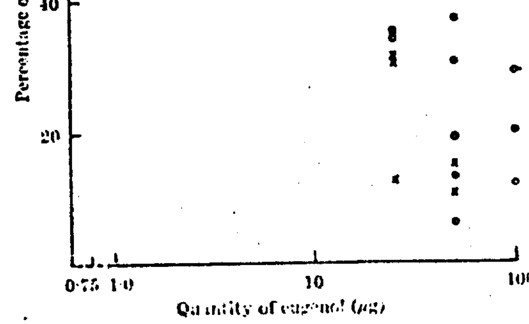

Fig. 1. The inhibitory effect of eugenol on the formation of *o*-aminophenolglucuronide by liver slices

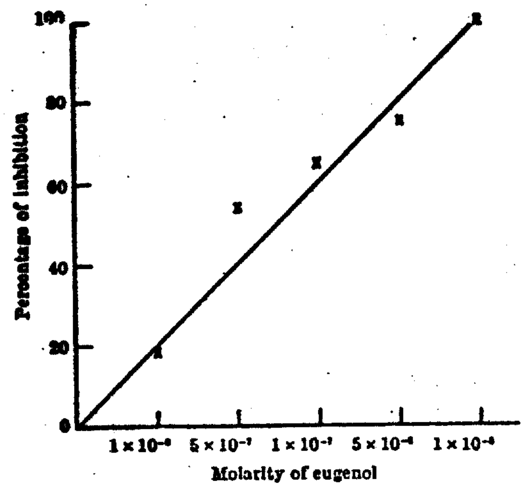

Fig. 2. The inhibitory effect of eugenol on the glucuronyl transferase activity of the liver

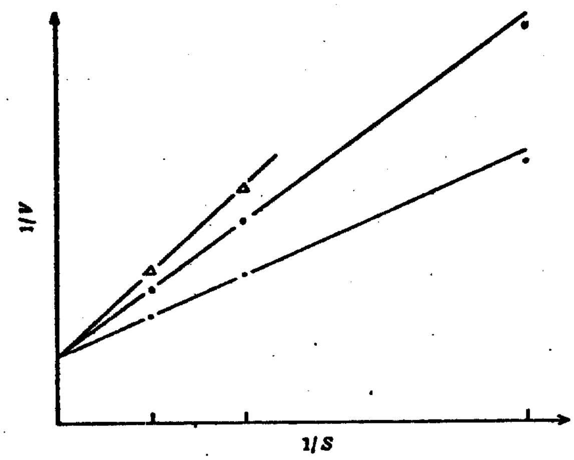

Fig. 3. The nature of the glucuronide conjugation inhibition by eugenol according to Lineweaver-Burk. △, $2\cdot5 \times 10^{-7}$ M eugenol; ○, 1×10^{-7} M eugenol; ●, control

however, to find the shortest effective time and lowest effective concentration.

When the specific effect of eugenol on the enzyme in the last stage of the conjugation reaction system (that is, on the glucuronyl transferase) was studied, it was found that for this enzyme the inhibition is K_{s_0} $2\cdot5 \times 10^{-7}$. The correlation between the degree of inhibition and the concentration of eugenol is shown in Fig. 2. The nature of this inhibition has been studied by preparing a Lineweaver-Burk plot of the results as shown in Fig. 3. It can be considered that the inhibition is of a competitive nature. The concentration of *p*-nitrophenol was also varied, but with the methods used no satisfactory results were obtained. The 'Triton X-100' was used in the experiments in order to increase the solubility of eugenol. It had no inhibitory effect at a concentration of 0·2 per cent. However, if the concentration was increased to more than 1 per cent, a very small inhibitory effect was obtained.

It has been possible to demonstrate the inhibition of β-D-glucosiduronic acid conjugation with eugenol. An important point in the reaction system is that UDPGA transferase would seem to be inhibited. It could be that this is not the only enzyme in the glucuronic acid conjugation system the activity of which is inhibited.

These results are in agreement with our earlier observations that eugenol can lower the quantity of salicylamide glucuronide formed in dogs when it is fed to them together with salicylamide[6]. It is difficult at present to correlate these observations with the mucinogenic effect of eugenol demonstrated by Hollander *et al.*

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Presynaptic Inhibition in Primate Lateral Geniculate Nucleus

SINCE their discovery[1-4], the effects of midbrain stimulation on excitability of the visual system have been studied extensively. Work on the squirrel monkey[5,6] has shown that the centrencephalic system exerts a powerful control over transmission through the lateral geniculate nucleus (LGN) for both photically and electrically elicited responses. Initial experiments showed facilitation following electrical stimulation of the mesencephalic reticular formation or natural alerting stimuli. Recent experiments, however, have shown that a brief period of inhibition precedes the facilitation and, as set forth below, strongly suggest that this inhibition is presynaptic in nature.

The phenomenon of presynaptic inhibition has been thoroughly reviewed by Eccles[7]. Among the characteristics which, in the mammalian central nervous system, reliably distinguish presynaptic from postsynaptic inhibition are susceptibility to different pharmacological agents. Strychnine, for instance, blocks most types of postsynaptic inhibition without affecting presynaptic inhibition, whereas picrotoxin strongly depresses the latter while leaving postsynaptic inhibition intact.

In the present investigation, 32 squirrel monkeys (*Saimiri sciureus*) were lightly anaesthetized; 20 with 'Diabutal' (sodium pentobarbital), 25 mg/kg, and 12 with 'Dial', 0·3 ml./kg. Pairs of electrodes made from 0·25 mm diameter 'Nichrome' wire were introduced into the optic tract (stereotaxic co-ordinates [8] : $A9$, $L5$, -1), optic radiation ($A1$, $L10$, $+3$), mesencephalic reticular

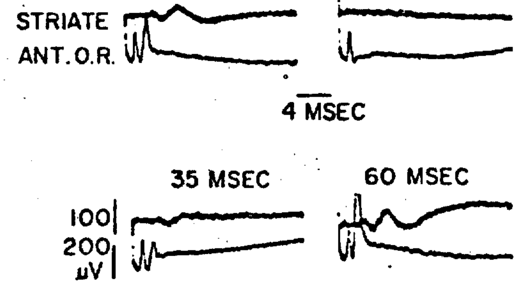

Fig. 1. Squirrel monkey R291. Control level of response in anterior optic radiation (Ant. O.R.) and striate cortex to stimulation of optic tract (10 V, 0·03 msec), bipolar electrodes. First peak in Ant. O.R., presynaptic or optic tract component; second peak, postsynaptic response conducted from LGN. Application of single pulse (15 V, 0·1 msec) to mesencephalic reticular formation 20 msec prior to optic tract stimulation abolishes responses in radiation and cortex. At 35-msec interval, inhibition not complete. Note at 60 msec, three-fold enhancement of postsynaptic responses. Presynaptic component unchanged throughout

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THE pH OF GASTRIC MUCOUS SECRETION[1, 2]

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Data at present available on the pH of gastric mucus are so scant that they contribute little to the sum of knowledge about the physicochemical characteristics of this exocrine secretion. Most of the reports (on material from dog, cat and human) state that it is alkaline to litmus, but a few investigators found an acid reaction to this indicator or even to Congo red. In patients with no free acid, Bucher (1) usually obtained pH's (colorimetric) in the range of 6.0 to 6.6, with an occasional value as high as 7.2. Mecholyl, injected subcutaneously into two fasting patients, yielded secretion with a pH (electrometric) of 8.9 (10). When administered iontophoretically, however, this pharmacological agent yielded pH's no higher than 8.3, but only a few values were reported (9). In dogs, the pH of mucus secreted spontaneously from both pyloric and fundic pouches was reported in the range 7.0 to 7.5 by Ivy and Oyama (7); Lim and Dott (8) found the same range for pyloric pouch juice, whereas Takata (11) reported 7.4 to 8.0. Similar material from cats had a pH of 8.4 (colorimetric), as reported by Gamble and McIver (2).

In the present investigation we determined the pH of a large number of specimens of mucous secretion, obtained from pouches of the gastric corpus in dogs. More than 575 values were obtained from both 'spontaneous' and chemically stimulated secretion, thus permitting of a statistical evaluation of the variability manifest in the work of previous investigators. Analysis of the opacity, consistency, and columnar cell content of a majority of these same specimens has already been presented elsewhere (5, 6).

METHODS

Almost all of the specimens of mucus employed for this study were collected from 10 Heidenhain pouch dogs; 15 of them were obtained from three other dogs with gastric explants prepared from the central portion of the greater curvature. Hence, the data relate to secretion from the gastric corpus—not the pyloric region. No experiment was ever started unless the mucosa was free of obvious parietal secretion, as evidenced by a pH of 6.0 or greater. The technique for establishing this condition, as well as all other details of collection procedure, have been described in a previous paper (5). Experiments were performed with

[1] Preliminary reports of this work were presented before the Federation of American Societies for Experimental Biology in 1941 and 1943. [Hollander and Felberg (3); Hollander and Stein (4)].

[2] This investigation was supported in part by grants from The Altman Foundation of New York and Wyeth Inc.

the following stimuli: 1) gentle massage of the mucosa with a soft rubber catheter for 3 to 5 minutes; 2) distilled water saturated with ether (no greater than six per cent concentration at 30°C.); 3) five per cent aqueous emulsion of clove oil; 4) aqueous eugenol emulsions in concentrations as high as five per cent; 5) ethyl alcohol, 50 per cent; 6) isotonic NaCl solution, 0.17 N; 7) hypertonic NaCl solution, 0.5 N; and 8) distilled water. A set of data on 9) 'spontaneous' secretion was also included. These last specimens were obtained either during experiments without direct stimulation or during preliminary periods preceding the application of one of the aforementioned stimuli. It may be assumed that none of the stimulating fluids exerted any chemical influence on the pH values for the following reasons: a) the stimulating fluid was aspirated from the pouch almost completely before the first specimen of secretion was collected; b) this specimen was discarded whenever it contained any visible amount of the stimulus; c) the stimulating fluids were unbuffered in all cases.

Electrometric pH determinations were made with a glass electrode. In general, no more than three minutes were required to attain constant readings, and these were found to persist for at least 15 minutes when a small series of specimens was studied for this purpose. Multiple determinations on any one specimen agreed within a range of ±0.02 pH, provided the specimens were shaken vigorously to effect homogenization before measurement. Without such shaking, duplicate determinations were liable to differ by as much as several tenths of a pH unit. After the reproducibility of this technique had been established, only single determinations were performed. Since we were concerned with the pH values of mucous secretion, which was presumably free of parietal secretion, all values above 4.00 were included in the tabulation for statistical analysis.

RESULTS

Combined data. A preliminary study of the frequency distribution for all 579 pH values, irrespective of stimulus, is presented in figure 1. The frequency graph is markedly skew, with a long tail on the acid side and a precipitous drop from 8.5 to the upper limit of the range, 9.2. The mean and standard deviations for the entire distribution are 7.65 and 1.08, respectively, but in view of the marked skewness of the graph, and the results of a breakdown of the data by stimuli, these two statistics possess no physiological significance. For purposes of analysis, the distribution was divided into two subranges at 6.8, where the flat tail of the graph terminates. It is noteworthy that more than 85 per cent of the values are in the upper subrange (table 1, line for combined data).

Data for individual stimuli. Next, the data for the total population were broken down according to stimulus, and the following statistics were calculated for each of these groups of data: the mean, its standard deviation ($S.D._M$) as well as that of the distribution (S.D.), and frequencies in the two pH subranges. These statistics are also presented in table 1. The stimuli are arranged in order of increasing mean pH—except for one per cent and two per cent eugenol, for which the difference in concentration has greater statistical significance than the difference in mean pH. Merely by inspection of the data it is apparent that the differences among these means are statistically significant in many instances,

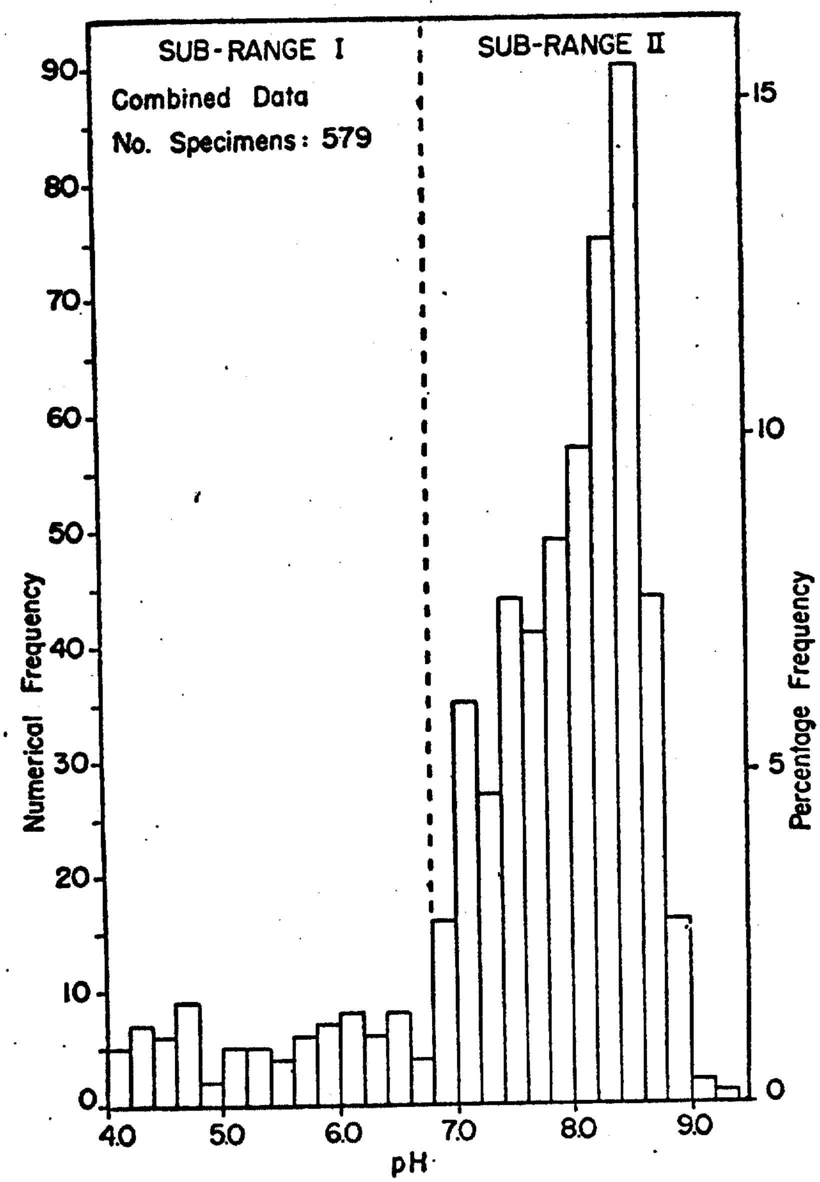
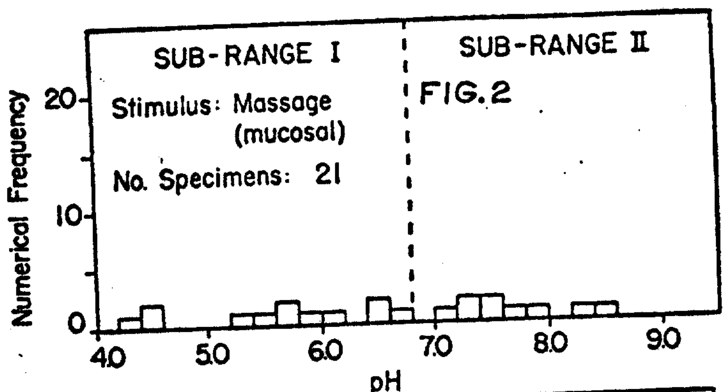


FIG. 1. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (combined data—all stimuli).

but not in all. In nine out of 12 cases, the difference between two *successive* means is only 0.2 pH unit or less; in two others it is about 0.3 and in the third 0.4. Because of this small variation in mean pH from stimulus to stimulus,

TABLE 1. Statistical data for pH values of gastric mucous secretion

Stimulus	Mean pH	S. D.	S. D.$_M$	pH range	Number of specimens: Sub-range 1 (pH 4.00–6.79)	Sub-range 2 (pH 6.80–9.22)	Total
Stimulus—group 1							
Massage (mucosal)	6.48	1.24	0.28	4.20–8.58	12 (57.1%)	9 (42.9%)	21
NaCl (0.17 N)	6.66	1.25	0.38	4.08–8.43	4 (33.3%)	8 (66.6%)	12
H_2O (distilled)	6.83	0.96	0.30	5.19–8.36	5 (45.4%)	6 (54.6%)	11
None (spontaneous)	6.90	1.33	0.14	4.00–9.07	30 (31.6%)	65 (68.4%)	95
Alcohol (50%)	7.02	1.01	0.17	4.20–8.22	8 (21.1%)	30 (78.9%)	38
Eugenol (0.25%)	7.35	1.32	0.31	4.35–8.60	5 (26.3%)	14 (73.7%)	19
Stimulus—group 2							
NaCl (0.5 N)	7.40	0.44	0.13	6.38–7.95	1 (7.7%)	12 (92.3%)	13
Ether (saturated)	7.63	0.88	0.09	4.50–8.83	10 (9.3%)	97 (90.7%)	107
Eugenol (0.5%)	7.97	0.56	0.12	5.70–8.53	1 (4.3%)	22 (95.7%)	23
Eugenol (1%)	8.04	0.58	0.09	6.24–8.65	4 (8.7%)	42 (91.3%)	46
Eugenol (2%)	8.03	0.57	0.07	4.76–8.87	1 (1.7%)	58 (98.3%)	59
Clove oil (5%)	8.10	0.27	0.05	7.28–8.47	0 (0%)	27 (100%)	27
Eugenol (5%)	8.51	0.44	0.04	4.69–9.22	1 (0.9%)	107 (99.1%)	108
Combined data (all stimuli)	7.65	1.08	0.04	4.00–9.22	82 (14.2%)	497 (85.8%)	579



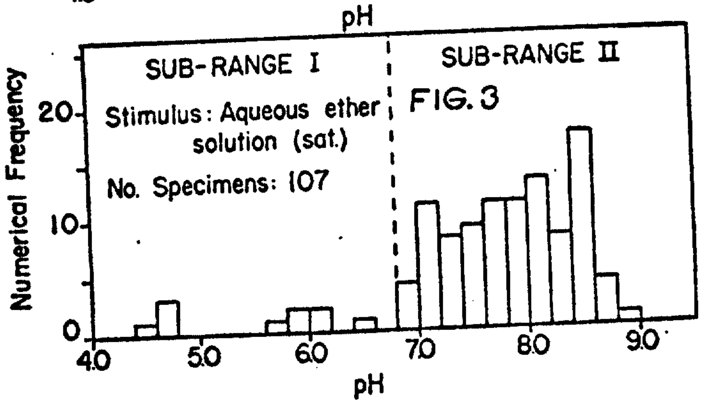


Fig. 2. Frequency distribution for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa by massage).

Fig. 3. Frequency distribution for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa with saturated aqueous ether solution).

these averages afford no basis for a classification of the stimuli. Such a classification, however, is suggested by the distribution of the data over the pH range. The values for mucosal massage, NaCl (0.17N), distilled water, 'spontaneous,' alcohol (50 per cent), and eugenol (0.25 per cent) extend more or less uniformly over the entire range. These are designated as stimulus-group 1. By way of illustration, the frequency graph for stimulation by mucosal massage is shown in figure 2. The data for the members of stimulus-group 2 show a well-defined concentration above pH 6.8, which is illustrated by the frequency graphs for ether, and eugenol (5 per cent) (figs. 3 and 4). For five of the stimuli in this group the number of specimens with pH's below 6.8 is no greater than one, and n the remainder the number does not exceed 10 per cent. In contrast with

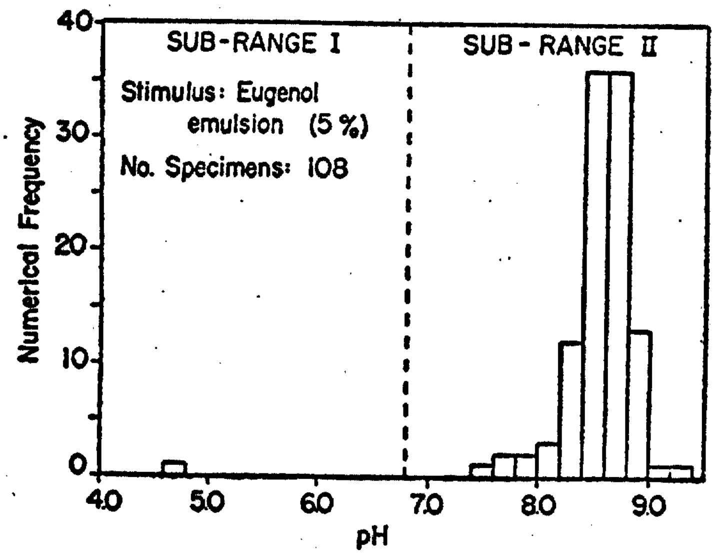


FIG. 4. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa with 5 per cent eugenol emulsion).

this, the proportion of pH values below this boundary value of 6.8 among the stimuli of group 1 varies between 21 and 57 per cent.

DISCUSSION

The marked skewness of the frequency distribution for the combined data (fig. 1), particularly the long flat tail from pH 4.0 to 6.8, suggests the presence of at least two different types of physiological fluid in what is generally called 'mucous secretion.' The first, with a very low pH value, is probably parietal secretion; the second, with a very high pH value, is true mucus and possibly a mucoid secretion. The peaks at pH 7.1 and 7.4 might result from a third type of fluid, of intermediate pH, e.g., transudate or exudate, but they are hardly high enough relative to the peak at pH 8.5 to constitute evidence of a tri- or bimodal distribution. However, sufficient support for the presence of transudate and exudate is provided by our previous studies to warrant their inclusion as a third possible factor in the interpretation of the present data.

Hence, if we hypothesize that gastric mucous secretion is a mixture of these three type of fluid in varying proportions, then the lower subrange of pH can be considered as containing values characterized by the dominance of parietal secretion, and the upper subrange by the dominance of mucus and mucoid secretions. Inasmuch as the frequency curve for the combined data has only one well-defined mode (pH 8.5), and this occurs in the upper subrange containing 86 per cent of all the values, it may be inferred that mucus and mucoid secretion are by far the most common of all these components. The occurrence of transudate and exudate is more difficult to define; these probably exert a secondary effect in both subranges, but particularly in the lower portion of subrange 2, below 7.8. The role of desquamated columnar cells has been neglected in this hypothesis. It seems likely that in the nondisrupted condition these cells exert little if any hydrogen ion effect. When disintegrated, however, they contribute not only mucus but also cytoplasm, which may be assumed to resemble transudate in its influence on pH.

Turning now to the breakdown of the data by stimulus, it is apparent that every stimulus herein reported yields some 'alkaline' mucus, but the potencies of these agents differ considerably. Actual data for the stimuli in group 2 (from table 1) reveal that 91 to 100 per cent of the specimens induced by eugenol (0.5–5 per cent), clove oil (5 per cent), ether (saturated) and NaCl (0.5N), consist predominantly of true mucus, with a pH of 6.8 or greater. Small amounts of parietal secretion, transudate or exudate may also be present in these specimens. On the other hand the agents of group 1—massage, isotonic saline, distilled water, 'spontaneous' and eugenol (0.25 per cent)—are the poorest mucus stimuli inasmuch as only 43 to 79 per cent of their specimens fall in the upper subrange.

The present study relates to the pH of mucous secretion as it occurs inside a gastric pouch. Since by far the major part of the unoperated viscus contains corpus mucosa, it was deemed essential that the pouches be prepared entirely from this region, rather than from the cardiac or pyloric portions of the stomach. Ultimately, however, we are concerned with the whole stomach rather than any such limited portion of it, but evidence available from the literature suggests that pyloric mucus has essentially the same pH as that from the corpus.

Whether the observations herein reported cast any light on the pH of mucus as it is formed within the cell cannot be stated as yet, because no attempt was made to control or compensate for the possible loss of CO_2 from the specimen, either while it was being collected from the pouch or thereafter. An observation of Wright and Florey (12) is noteworthy in this connection. They reported that the pH of the viscous secretion of the cat's colon, resulting from faradic stimulation of the peripheral ends of the nervi erigentes, is at first 8.3 to 8.4 but increases to 9.1 to 9.2 on exposure to air. In the present work, it was noted that the pH of different portions of any one specimen become essentially identical if the specimen is well shaken. The uniformity so effected was ascribed to 'homogenization' of the specimen, but it may well be that the initial lack of uniformity was a result of irregular loss of CO_2 prior to shaking. Prevention of CO_2 loss before the specimen enters the collection tube is impossible with the usual procedures for collecting the gastro-intestinal secretions. However, correction for such loss may be possible by equilibration of the specimen immediately before the pH is measured, using air containing CO_2 at its partial pressure in alveolar air. Such studies with gastric mucous secretion are now under way.

SUMMARY

Electrometric pHs were determined for 579 specimens of gastric mucous secretion collected from Heidenhain (corpus) pouch dogs. For this purpose, 12 topical stimuli were employed. The range of these pH values was 4.00 to 9.22, with a mean of 7.65 and a standard deviation of 1.08. Division of the data into two subranges of pH revealed that 82 (14 per cent) of the values fell in the lower interval (pH 4.00 to 6.79) and 497 (86 per cent) in the higher interval (6.80 to 9.22). It is inferred that the pH of this latter group is determined predominantly by true mucus, whereas the pH of the former group is influenced in considerable degree by the acid parietal secretion. Division of the data into 13 groups according to stimulus (including spontaneous secretion) revealed a gradual but extensive variation in the mean pHs for the individual groups. However, only aqueous emulsions of clover oil (5 per cent) and eugenol (0.5 to 5 per cent) yielded secretion with mean pHs greater than 8.0. Furthermore, 97 per cent of all the specimens obtained with these stimuli fall in the upper subrange, with pHs greater than 6.8. These observations support the conclusion previously reached by us that clove oil and eugenol are considerably more effective stimuli for the collection of gastric mucus than any others in the series.

The findings herein reported cast no light upon the pH of pure mucus as it is ejected by the surface epithelial cells, because of the loss of CO_2 from the specimen between the time of secretion and the time of pH measurement. Nevertheless, the data herein reported do indicate the pH which may be encountered in gastric pouches, and therefore presumably in the lumen of the intact resting stomach, under the influence of various mucus stimuli. This pH is usually above 7.4 and may even be greater than 9.0—probably the highest value encountered in the living mammalian organism.

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HISTOLOGICAL STUDY OF THE DESTRUCTION AND REGENERATION OF THE GASTRIC MUCOUS BARRIER FOLLOWING APPLICATION OF EUGENOL. PRELIMINARY REPORT [1 2 3]

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INTRODUCTION

At the Gastric Cancer Conference in 1946 (*1*), our group of investigators reported on the changes in character of gastric mucous secretion which result from the repeated application to the gastric mucosa of dogs of a mucigogue-desquamating agent. This physiological study had been conducted with Heidenhain-pouch dogs, using an aqueous emulsion of eugenol as the stimulus. During the last year, these experiments were repeated with this modification: the experiments were terminated at various stages of progress by killing the dogs, in order to study successive histological changes in the surface of the gastric mucosa. The present report is a preliminary statement concerning the results of these experiments. The immediate objectives of this line of investigation are: (1) to trace the extent of functional exhaustion and recovery of the mucous secreting cells; (2) to determine the extent of desquamation of the surface epithelium; and (3) to ascertain the changes in character and the extent of replacement of the mucous cells themselves. In addition, these studies promise to yield information on the cytological processes of mucous secretion in the stomach and, possibly, evidence concerning the early stages in the experimental development of gastritis.

The broad plan of the original physiological experiments is as follows: after a considerable control period, a 5-percent aqueous eugenol emulsion was placed in the pouch for 15 minutes and then aspirated completely, following which four 30-minute specimens of secretion were collected. The dog was allowed a rest period of about 15 minutes, and then a second cycle of stimulation and collection was started. These cycles were repeated until the secretion had changed in character from a true viscous mucus to a fluid which was mostly inflammatory exudate, i. e., until it was inferred that the mucous barrier was completely exhausted. Although this change usually required five successive cycles, an additional one or two cycles were added to insure completeness of the transformation in

[1] Presented before the Fourth Conference on Gastric Cancer, held at Stanford University School of Medicine, San Francisco, December 13, 1948.

[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The authors wish to acknowledge the participation of Dr. B. P. Sonnenblick in the early part of this investigation.

character of the secretion. Thus, these fatigue experiments entailed six or seven cycles in all and a duration of 15 to 18 hours. The animal was then returned to its cage for a recovery period of 30–36 hours after which the eugenol was again applied as before, until the mucus-secreting apparatus was once more fatigued. This first follow-up experiment, designed to reveal the extent of tissue recovery which occurred during 1½ days of rest, usually lasted for only one or two cycles before the secretion again became a bloody serous fluid. Subsequently, a second follow-up experiment was performed, but this time the intervening recovery period lasted 2 to 3 months. As a result, it then required three or four cycles to fatigue the mucous barrier, indicating that this structure had recovered extensively, but it had not yet returned to its original state prior to the very first application of eugenol. On the basis of the changes in several physicochemical properties of the secretion and in the microscopic appearance of smears from the numerous specimens collected in the course of these studies, inferences were drawn regarding the progressive changes which had probably occurred in the gastric mucous barrier. In order to confirm these inferences, the present series of experiments was undertaken.

EXPERIMENTAL PROCEDURES

The general plan of the present experiments, as well as the details of technique, were essentially the same as before (*1*). This time, however, the individual experiments were interrupted at various stages by killing the animals. Pieces of tissue were then removed from pouch and residual stomach and placed in Bouin's fixative as rapidly as possible. Sections were stained with haematoxylin and eosin and with toluidine blue. A total of 19 pouch dogs were prepared for this study; of these, 1 died, and 2 were discarded for reasons unrelated to the project. None of the dogs was ever used for any purpose but this, and a period of 2–3 months was always allowed to elapse between preparation of the pouch and the initial fatigue experiment. In addition to the residual stomachs of all the animals, 4 of the pouch dogs served solely as controls and were killed 2 to 3 months after operation without having received any eugenol treatment whatever. The initial fatigue experiments were terminated at the following approximate times after removal of the stimulus: ¼, ½, 1¼, and 2¼ hours following a single application of eugenol; ½, 1½, and 2 hours following the third such application; and 2 and 36 hours after the seventh. In addition to these dogs which were killed in the course of the initial fatigue series, there were 3 which were subjected to subsequent follow-up experiments. One of these animals was sacrificed 3 months after the first follow-up experiment had been terminated. The other 2 dogs were subjected to a second follow-up experiment 5 months after the first; these were allowed final recovery periods of 3 weeks and 4 months, respectively, and were then killed. One to 8 pieces of the residual stomach were taken from all 16 animals for control purposes, in addition to specimens from the pouches of the 4 untreated dogs. Thus, by varying the number of eugenol applications and the time which was allowed to elapse between the last such application and the death of the animal, it was possible to study a variety of stages of impairment and recovery of the mucous barrier. The following are the results as revealed by some of the more obvious differences in structure of the tissue surface.

RESULTS

Controls

Sections of untreated tissue (Pl. 56, fig. 1, CA 75–8) generally reveal surface mucus as very thin short ribbons or small amorphous masses of metachromatic material, interspersed with areas of normochromatic substance. The crypts contain much less mucus than is present on the surface of the mucosa, and in both regions the material is nearly, if not entirely, free of columnar cells. In depth, the crypts may be long or of only moderate size relative to the length of the entire gastric gland, and these two varieties occur with about equal frequency. There are virtually no stretches of mucosa entirely free of crypts, and only rarely does one encounter a shallow crypt or a depression in the surface suggestive of the remnant of a foveola which had been removed by desquamation of its columnar epithelium. In fact, the control specimens give almost no evidence whatever of active desquamation; even gaps in the rows of columnar cells, indicative of cells which have been shed and not yet replaced, are encountered only rarely, and these are usually no more than one cell wide. Ranks or palisades and single columnar cells are seen within the adherent layer of surface mucus only occasionally, if at all. Finally, both within the crypts and on the surface of the mucosa between the crypt mouths, practically all of the mucous cells are tall columnar cells with large thecae containing metachromatic material. Occasionally a parietal cell, white blood cells, or a few erythrocytes may be seen wedged in or just beneath this layer of surface epithelium.

Destruction of the Mucous Barrier

Successive stages in the degenerative process were traced by three experiments which were terminated in the first, third, and seventh cycles of the fatigue series, respectively. In the first two of these experiments, ¼ hour elapsed following removal of eugenol; in the last, this time interval was 2 hours.

A quarter of an hour following the first application of eugenol (Pl. 56, fig. 2, CA 75–2), the pouch sections already gave evidence of significant changes from the controls, and this observation is supported by the over-all picture at the end of the first half hour (Pl. 56, fig. 3, CA 76). Mucus in considerable amounts is extensively distributed over the surface, and even within the crypts, though here again to a lesser degree. Although some of this mucus is acellular, especially in the crypts, most of it contains numerous columnar cells, singly and in ranks, and also some parietal cells, neck chief cells, white blood cells, erythrocytes, and other elements which have already been reported as occurring in mucous smears. Although the proportion of short crypts is about the same as in the controls, really deep ones are rarely encountered, whereas shallow depressions in the mucosal surface are notably present. Furthermore, there are now a great many more gaps between the columnar cells than were found in the untreated specimens, and these spaces are often six or more cells in width. There can be no doubt that these gaps result from desquamation rather than technical defects, because large and small ranks of columnar cells are dispersed in the extracellular mucus immediately adjacent to them, and palisades of these cells can often be seen in the process of being cast off. In short, these two experiments give ample evidence of a progressive increase in both secretory and desquamatory responses during the first ¾ of an hour of cycle A in the fatigue experiment.

In the third cycle (Pl. 56, fig. 4, CA 64), 30 minutes after removal of the eugenol, the destruction of the mucous barrier is seen to have progressed even further. The amount of extracellular mucus is now markedly decreased, and its cell content somewhat less so, but signs of active desquamation are still present. This secretion is only slightly metachromatic and it contains some exudative material with traces of blood. The proportion of surface which is crypt-free or contains only shallow depressions is even greater than before, and the gaps are now sufficiently wide and numerous as to suggest actual denudation with exposure of the underlying connective tissue. Although tall columnar cells with metachromatically staining thecae are still present, there are now numerous columnar cells which are theca-free and also some flat cells not encountered in the experiments described above. These cells will be considered in detail below.

Finally, by the end of the seventh cycle (Pl. 57, fig. 5, CA 68) definitely formed crypts have disappeared to a very large extent. Shallow depressions predominate, though regions entirely devoid of crypts are also numerous. The surface of the tissue appears to have lost most of its mucus-containing columnar cells, and there are now many regions which are completely denuded of surface epithelium. It is only in the small proportion of remaining crypts that metachromatically staining columnar cells still occur. Signs of active desquamation are virtually absent, and the surface is now covered with a bloody inflammatory exudate containing very little metachromatic mucin and very few mucous cells. Parietal cells are also seen in this extracellular layer.

In this way we have traced the progressive destruction of the mucous barrier through seven cycles of the fatigue experiment, to the stage where true mucus secretion and desquamation no longer occur. In general, the histological evidence of this destruction is in accord with the changes in character of the mucus secretion which were encountered in our previous work.

Regeneration of the Mucous Barrier

Our earlier physiological experiments gave evidence of a recovery process as well as a destructive one. Therefore, we next studied the course of events in three groups of dogs, in each of which all the animals received the same number of 15-minute applications of eugenol emulsion but were killed at different times after the end of such treatment. These three groups consisted of four animals killed in the course of the first cycle of the fatigue experiment, three during the third cycle, and five dogs after the end of a fatigue or of a follow-up experiment.

The existence of a reparative process and the speed with which it occurs are well illustrated by the group of four experiments which were terminated during the first cycle at approximately ¼, ½, 1¼, and 2¼ hours after removal of the eugenol. As noted previously, there was no great difference in the character of the surface epithelium of the first two of these experiments (CA 75 and 76). The epithelial elements present in the surface layer are tall columnar cells, with an occasional parietal and neck-chief cell in or immediately beneath the surface. However, there is one suggestive difference between these two experiments not shown in the photomicrographs. The proportion of mucosal surface that was free of crypts or contained only shallow depressions was less in the half-hour experiment than in the quarter-hour one. Further evidence of this was manifest at the end of an hour (Pl. 57, fig. 6, CA 50) when complete crypts occupied a major part of the entire surface, whereas the proportion of regions with shallow or no depressions was greatly diminished. This reappearance of crypts was itself indicative of a reparative process, but, in addition, the gaps between columnar cells occurred much less frequently than in the two earlier experiments of this cycle and were generally of smaller size.

The mechanism of this reparative process is evidently linked to the appearance of new cells in the surface layer, fusiform and flat epithelial cells which contain no metachromatic material, even though nearby columnar cells have already started to regenerate their intracellular mucus. Flat cells occurred for the first time in the last-mentioned experiment, usually in small groups on the surface of the mucosa. Frequently, they occurred at the end of a row of columnar cells which ranged in size progressively from a tall variety through a series of shorter columnar cells, and finally ended in these flat elements. Not only do the cells in these transition series vary in over-all height, but they may also show a gradation in regard to their content of intracellular mucin, from large metachromatic thecae at one end of the series to theca- and mucin-free cells at the other. The presence of even an occasional one of these transition series 70 minutes after eugenol treatment is highly suggestive of a resurfacing of exposed mucosa with newly formed cells, which takes place with amazing rapidity (as reported by Grant (2)) probably in advance of crypt reformation. The fact that both mucus secretion drained from the pouch during this time (specimen 4 of the cycle) and extracellular mucus which remained adherent to the mucosal surface in these tissue sections contained significant numbers of columnar cells may be indicative of continuing desquamation in spite of a simultaneous regenerative process. It seems more likely, however, that all traces of eugenol emulsion would have been carried off from the surface of the mucosa by the highly cellular mucus formed during the first half hour of secretion and that most of the shed epithelium would have been loosened from its basement membrane before the time when this experiment was stopped by sacrifice of the animal. Therefore, the desquamated columnar cells found at this stage of the cycle must have been shed for the most part during an earlier stage. They remained adherent to the mucosa because of the high viscosity and adhesiveness of the mucus in which they were embedded.

One hour later, that is, at the end of the first cycle and about 2 hours after removal of the eugenol (Pl. 57, fig. 7, CA 49), the tissue sections give evidence of still further diminution in mucus output. Also, the content of desquamated columnar cells in this mucus was almost negligible. The proportion of mucosal surface which was devoid of complete crypts was less than in the previous experiment, and extensive denuded regions no longer occurred. Gaps between columnar cells were still present, but their incidence and size were no greater than in some of the control specimens. Thus, although reformation of crypts is still very far from complete, the denuded areas were again covered by some form of epithelium. As in the previous experiments, tall columnar cells predominated, but their average thecal size and mucus content now appeared to be greater. Flat cells were also present in small groups and in transition series, giving evidence that resurfacing of the mucosa was still going on actively at this time.

These regenerative phenomena were all observed in the first group of animals, all of which received only one application of eugenol. The other two groups of experiments (one after three applications, the other after complete fatigue) revealed even greater incidence of these flat cells than was encountered in the experiments just discussed. This was particularly marked at certain stages of the third and seventh cycles (figs. 4 and 5, CA 64 and 68) wherein denudation followed by resurfacing was noticeably more extensive than in the first cycle. Thirty-six and a half hours after the seventh cycle (Pl. 57, fig. 8, CA 69), resurfacing of the mucosa with tall columnar cells appeared to be entirely complete; flat cells were no longer evident in any of the sections. These columnar cells generally contained thecae which were relatively large and full of deeply metachromatic mucus. Crypt reformation also made considerable progress, for although the entire surface was void of crypts 2 hours after the seventh application of eugenol (Exp. CA 68), areas free of fully-developed crypts constituted less than half of the surface after 36½ hours of recovery (Exp. CA 69). It is significant also, that there were more short crypts at this time than long ones, and they all tended to be irregular in shape and even tortuous. Follow-up experiments with recovery periods of 3 weeks to 4 months (Exps. CA 42, 43, and 44) manifested continuing regeneration of the mucous barrier. In fact, when the interval of recovery was 3 to 4 months, the regeneration appeared to be complete, for crypt-free areas were virtually absent.

Even though physiological activity may be restored during the two recovery periods following complete destruction of the mucous barrier, such recovery of secretory function is not necessarily complete, and this is generally corroborated by the extent of structural recovery found in the present investigation. Thus, in the first follow-up experiments, only one or two cycles were required to effect a radical change in the character of the secretion, from a true viscous mucus to a serous exudate, and in the second follow-up experiments, four cycles were usually required to attain the same end result. In the present morphological study, tissues taken at a time corresponding to the beginning of the first follow-up experiment (1½ days after complete fatigue, Exp. CA 69) revealed only partial replacement of crypt structure, as stated in the previous paragraph. On the other hand, specimens taken 3 months after the first follow-up experiment (Exp. CA 44), which time corresponds to the beginning of the second follow-up experiment, showed an almost completely reconstructed mucosa. Thus, the explanation originally postulated to account for the difference between the first and second follow-up experiments, in regard to the number of cycles required for exhaustion following recovery periods of different durations, is now substantiated by the variations in mucosal regeneration seen in the present study.

CONCLUSION

The histological study, of which this is a preliminary report, presents evidence of the changes in the gastric mucous barrier which result from the repeated application of a mucigogue-desquamatory agent (aqueous eugenol emulsion) to the mucosa of Heidenhain pouches in dogs. In general, the histological evidence is in accord with the results of our previous study on the changes in microscopic and physicochemical character of the mucus secretion collected in the course of such experiments. This correlation of the physiological and histological observations obtains with regard to the impairment and virtually complete destruction of the mucous barrier and also with regard to its subsequent regeneration. Successive applications of the eugenol emulsion effect a progressive removal of the columnar cells from the surface of the mucosa and then from the crypts themselves, until the connective tissue matrix containing the collecting tubules of the glands is exposed. The regenerative process can be divided into three stages: (1) a preliminary resurfacing of denuded mucosa with flat and fusiform-shaped cells, which is already evident 30–60 minutes after removal of eugenol from the pouch; (2) the transformation of these new cells into low and tall columnar cells; and (3) the reformation of crypts in these areas of smoothly resurfaced mucosa. This entire process has been found to occur within 36 hours following the removal of the mucosa as far down as the bottoms of the foveolae. The surprising rapidity with which these changes take place serves to explain many aspects of the original physiological experiments which were not fully understood before. Data from these experiments indicate the conditions of pretreatment of the gastric mucosa with eugenol which are most suitable for attempts at carcinogenesis by topical application of known carcinogens after impairment of the mucous barrier. We already know the optimum duration of eugenol treatment and of posteugenol recovery in order that (1) the protective mucous barrier be impaired extensively but not completely, and (2) that resurfacing of the mucosa be active so as to insure the presence of newly formed cells, presumably in a state of maximum susceptibility to cancer induction. Experiments on rats and mice, analogous to these on dogs, are now in progress, and as soon as they have been completed, carcinogenetic experiments will be initiated.

The experiments described present many problems which require further minute study of the tissue sections. Among these are: the types of cell and mechanism involved in the resurfacing process; the importance of mitosis in the formation of new cells for reconstitution of the mucosa denuded of its crypts and surface columnar cells; the possible occurrence of a progressive gastritis as indicated by lymph follicles, cysts, and tortuous crypts; and the cellular process of mucus secretion, apart from the concomitant desquamation. These are problems for a histopathologist rather than a physiologist, and we now plan investigation in collaboration with such a specialist.

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PLATE 56.

FIGURE 1.—Exp. CA 75–8. Control tissue. Residual stomach. No eugenol application. × 100.

FIGURE 2.—Exp. CA 75–2. Initial fatigue series. Pouch tissue 14 minutes after first eugenol application. × 100.

FIGURE 3.—Exp. CA 76–1. Initial fatigue series. Pouch tissue 29 minutes after first eugenol application. × 100.

FIGURE 4.—Exp. CA 64–5. Initial fatigue series. Pouch tissue 27 minutes after third eugenol application. × 100.

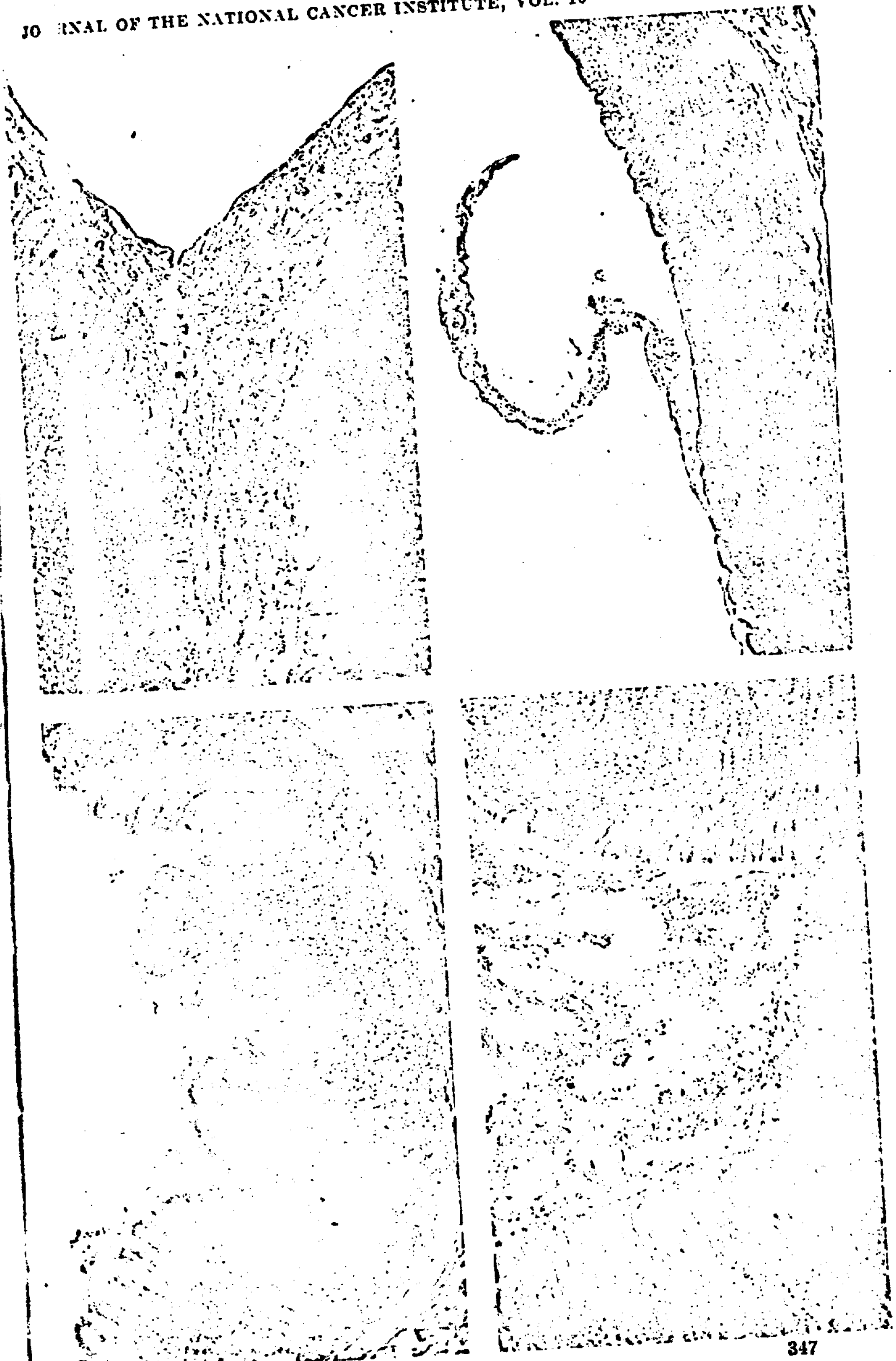

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PLATE 57

FIGURE 5.—Exp. CA 68-1. Initial fatigue series. Pouch tissue 120 minutes after seventh eugenol application. × 100.

FIGURE 6.—Exp. CA 50-2. Initial fatigue series. Pouch tissue 70 minutes after first eugenol application. × 100.

FIGURE 7.—Exp. CA 49-6. Initial fatigue series. Pouch tissue 130 minutes after first eugenol application. × 100.

FIGURE 8.—Exp. CA 69-1. Recovery period. Pouch tissue 36½ hours following initial fatigue series. × 100.

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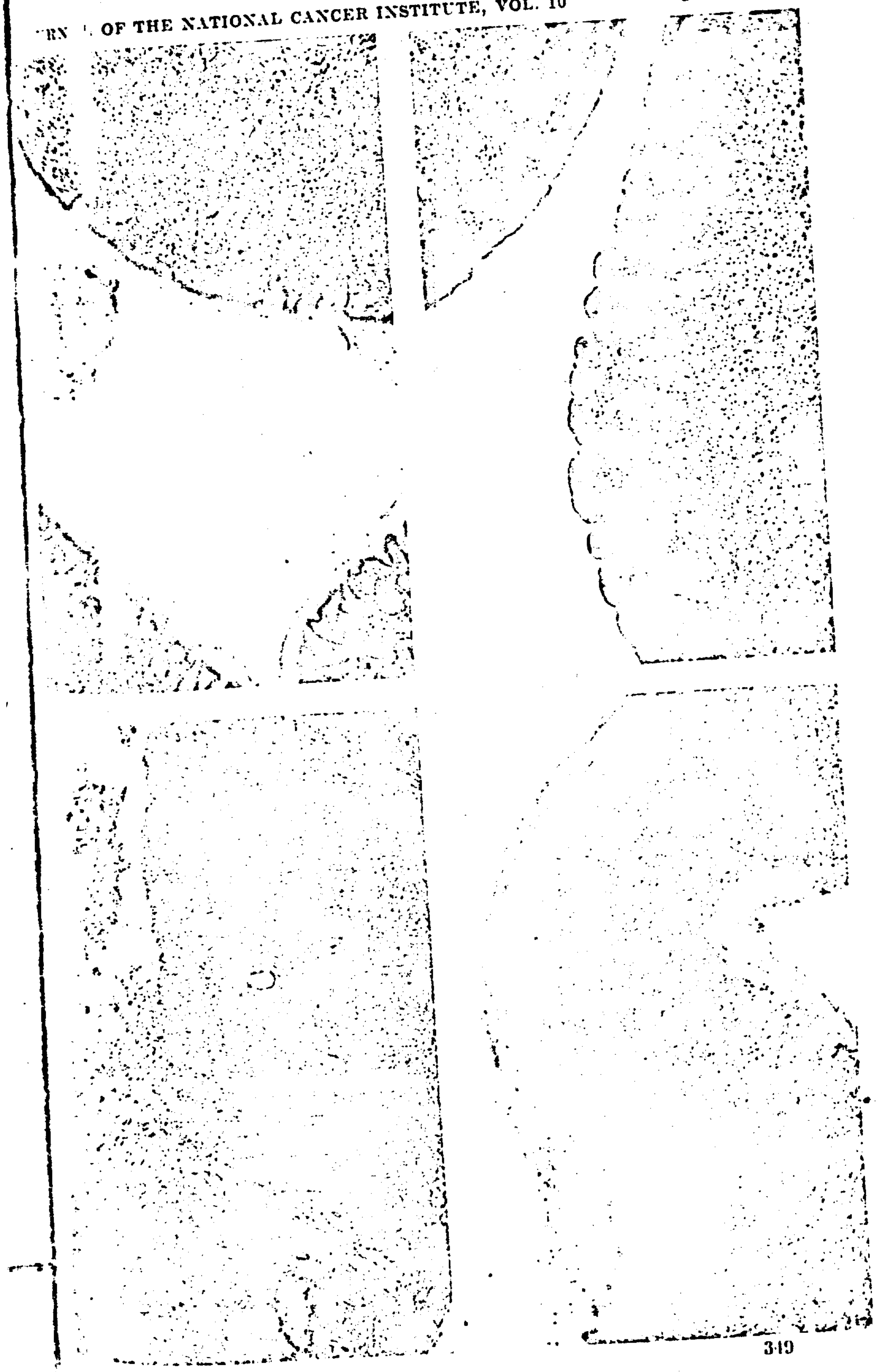

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undifferentiated sarcoma, one osteogenic sarcoma and one plasmocytoma. Metastases occurred in 3 instances. Among the tumors derived by transplantation there were 63 fibrosarcomas, and a few other miscellaneous growths. Variation in differentiation and growth rate was noted in different generations.

Discussion. We have arrived at no conclusion as to the nature of the carcinogenic agent in these experiments. Attention is called to the fact that the procedure described is one of the simplest known for the production of sarcomas. In this connection the production of rat sarcomas by Turner[3] by embedding disks of bakelite (phenol-formaldehyde) is of interest.

In the course of certain surgical procedures, cellophane has been used as a covering or sheath,[4] and left in the human body. We are not acquainted with any reports of the development of sarcoma in man subsequent to its use, but this possibility should not be forgotten.

Conclusions. 1. Sarcomas were induced in albino rats by the insertion of regenerated cellulose film either subcutaneously or by wrapping it around one kidney.

2. These tumors occurred in about 35% of the rats surviving the operation more than 11 months.

3. The tumors were transplantable.

4. This is a simple method for inducing sarcomas experimentally.

5. The foregoing results in rats should be taken into consideration in the surgical use of cellophane in man.

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Eugenol as a Stimulus for Gastric Mucous Secretion*†‡

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In the course of investigations in this laboratory on the characteristics of gastric mucus, various types of stimulation have been studied. These included subcutaneous injection of pilocarpine, gentle mucosal massage, and topical application of a number of aqueous solutions and emulsions including ether (saturated), ethyl alcohol (50%), isotonic NaCl (0.17 N), hypertonic NaCl (0.5 N), clove oil (5%), mustard oil (1%), and distilled water.[1,2,3] Among the several objectives of these investigations was the discovery of a mucus-stimulating agent which induces no parietal secretion and therefore might be adopted as a standard stimulus for further work on the physiology of mucus secretion. Of the nine stimuli already studied, the 5% clove oil-water emulsion was clearly the most suitable for the purpose, as evidenced by the following characteristics of specimens obtained after a single application of this agent: (a) high viscosity, (b) high pH,[4] and (c) a large total volume relative

* A preliminary report of this work was transmitted to the XVII International Physiological Congress (Hollander, F., and Lauber, F. U., *Communications XVII Internat. Physiol. Cong.*, 1947, p. 155).

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‡ The authors wish to express their thanks to Dr. Sophya Lazard for conducting some of the experiments.

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TABLE I.
Data for Several Characteristics of Gastric Pouch Mucus.
(Stimuli: aqueous emulsions of clove oil and eugenol.)

			Stimuli			
			Eugenol			
			Low concentration (¼-½%)	Intermediate concentration (1-2%)	High concentration (5%)	Clove oil, (5%)
Volume per exper.		Mean	1.7	4.1	6.4	2.0
		Stand. Dev.	1.89	3.75	3.15	1.10
		No. of exper.	13	21	65	9
pH		Range	4.35-8.60	4.76-8.87	7.53-9.22	7.28-8.47
		Mean	7.69	8.03	8.51	8.10
		Stand. Dev.	1.03	0.57	0.44	0.27
		No. of specimens	42	105	108	27
Consistency	No. (%) of specimens	Viscous	29 (64.4%)	64 (59.8%)	92 (64.8%)	27 (96.4%)
		Fluid	14 (31.1%)	30 (28.0%)	32 (22.5%)	1 (3.6%)
		Mixed	2 (4.4%)	13 (12.1%)	18 (12.7%)	0 (0.0%)
		Total	45	107	142	28
Opacity	No. (%) of specimens	Opaque	26 (59.1%)	54 (56.3%)	104 (81.9%)	26 (96.3%)
		*Non-opaque	18 (40.9%)	42 (43.7%)	23 (18.1%)	1 (3.7%)
		Total	44	96	127	27
Columnar Cells	No. (%) of specimens	Many	28 (68.3%)	76 (72.4%)	35 (83.3%)	19 (95.0%)
		†Few	13 (31.7%)	29 (27.6%)	7 (16.7%)	1 (5.0%)
		Total	41	105	42	20

* Non-opaque = transparent or translucent.
† Few cells = none or a small number of cells per field.

to that secreted spontaneously in the same period of time.

The one disadvantage in the use of clove oil as a standard stimulus arises from its being a mixture of several chemical compounds in variable proportions, rather than a single chemical individual. However, the chief component of this essential oil is eugenol (4-allyl-2-methoxy phenol) which comprises 82-87% of the mixture. Hence, it seemed that this compound may be responsible for a major part of the mucus-stimulating action of the clove oil, and, therefore, better suited for use as a standard mucus stimulus. We have investigated the secretory response of the gastric mucosa to topical application of aqueous emulsions of pure eugenol, and the results are presented in this report.

Procedure. The experimental technique was the same as that used previously for studying the action of clove oil and the other stimuli.[3] The eugenol emulsions were prepared in concentrations ranging from ¼ to 5%. Acacia (5%) was used as an emulsion stabilizer in the earlier experiments; later this was replaced by Tergitol Penetrant-4 (1/40%) with equally good results.§ Control experiments with these substances alone showed that they exert no mucus-stimulating action at these concentrations; the fluid obtained after their application was the same as spontaneously secreted mucus in appearance and rate of formation. As in our previous work, the eugenol stimulus was administered only after the pH of the control (pre-stimulation) specimens had risen above 6.0. In general, collection of the stimulated specimens was continued until the rate of secretion fell to its control value. The pH's were determined electrometrically, with a glass electrode, and were reproducible to ±0.02 of a unit. A total of 99 experiments (*i.e.*, stimulations) was performed with eugenol emulsions, using 9 dogs with Heidenhain pouches.

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§ Tergitol Penetrants were kindly supplied by the Carbide and Carbon Chemicals Corp., New York City.

Results. The data on characteristics of mucus specimens obtained after eugenol and 5% clove oil emulsions are presented in Table I. Because of the small frequencies in some of the groups of data, the values for ¼ and ½% eugenol were combined into a single group designated "low concentration," and those for 1 and 2% into an "intermediate concentration" group. The resulting increase in the number of specimens in each group augments the chance of obtaining a significant statistical evaluation of the observations. The frequency of the 5% eugenol data being large, these were retained alone as the "high concentration" group.

Total volume of mucus secreted in response to a single application of stimulus. The mean volume of secretion per experiment increases with the concentration of eugenol in the stimulus, from 1.7 ml for the "low concentration" group to 6.4 ml for the "high concentration." This latter value, for the 5% eugenol concentration, is significantly greater‖ than that for 5% clove oil (2.9 ml per experiment). The datum for 1-2% eugenol ("intermediate" group) is also greater than that for 5% clove oil but the difference is not significant.

pH. As shown in Table I, the mean pH of eugenol-stimulated specimens increases with increasing concentration of the stimulus, the differences between the several groups being significant at or below the 1% level of probability.¶ Corresponding to this, there is an elevation of both the upper and lower limits of the range, but the data become increasingly homogeneous, as evidenced by a decrease in the range itself and in the standard deviation. The mean pH for 5% clove oil is significantly lower** than that of eugenol of the same concentration but is essentially the same as that for 1-2% eugenol. Since the 5% eugenol emulsion gives a higher mean pH and a lower standard deviation than any of the other stimuli which we have previously studied,[5] it is the most effective agent encountered to date for evoking an alkaline response from the stomach.

Consistency, opacity, and columnar cell content. Qualitatively, these physical characteristics of mucus were essentially the same for eugenol as for all the other topical stimuli, except mustard oil emulsion. Some of the specimens were distinctly fluid; others jelly-like or of intermediate viscosity. Some were transparent or translucent; others opaque. Some were cell-free or contained only cellular detritus; others contained many columnar cells—singly, or in ranks and clumps. In microscopic appearance, after being stained with toluidine blue, the eugenol-mucus smears were indistinguishable from those of clove oil-mucus. Numerically, the percentage incidences for the three categories of consistency shown in Table I are essentially the same for all 3 concentrations of eugenol; clove oil, however, gives a markedly higher percentage of viscous specimens. For opacity, the percentages of opaque specimens in the low and intermediate concentration groups are almost identical; whereas the value for 5% eugenol is considerably higher than these, and that for clove oil even greater.†† The values for columnar cell content show this same trend, although the differences are not significant statistically by the χ^2-test ($P > 1\%$).

The consistently higher position of clove oil over eugenol, in regard to all three of these physical characteristics, is probably the result of the action of one or more compounds in the essential oil other than eugenol. In addition to the latter, clove oil contains vanillin, methyl alcohol, furfural, caryophyllene, acetyl

‖ This difference is significant at the 1% level of probability; $t = 3.3$, $n = 72$, $P = 0.18\%$.

¶ The difference between the mean-pH's for the low and intermediate concentrations gave the following statistics in the t-test: $t = 2.6$, $n = 145$, $P = 1\%$. For the intermediate and high concentration groups, $t = 6.9$, $n = 211$, $P < 0.01\%$.

** $t = 4.8$, $n = 133$, $P < 0.01\%$.

[5] Hollander, F., Lauber, F. U., and Stein, J. J., in preparation.

†† These differences in relative frequencies are statistically significant at the 1% level of probability, as shown by the χ^2-test. For the first of these—with categories of opaque and non-opaque specimens, and "high" and "intermediate" concentrations of eugenol—$\chi^2 = 17.4$, $n = 1$, $P < 0.01\%$. For the second—with categories of opaque and non-opaque specimens, and 5% clove oil and "intermediate" concentrations of eugenol—$\chi^2 = 14.8$, $n = 1$, $P = 0.01\%$.

eugenol, and eugenol acetyl salicylate,[6] and it is possible that some of these substances are particularly potent as desquamating agents, even in small quantities.

Summary. The characteristics of gastric mucous secretion, stimulated by topical application of aqueous eugenol emulsion in several concentrations, have been investigated on 9 Heidenhain pouch dogs. A 5% emulsion of clove oil, which had previously been found to be superior to all other mucus stimuli, was used as a basis of reference for determining the secretory value of eugenol. It was found that 5% eugenol yields larger volumes of secretion, with a higher pH, than the clove oil. The percentages of specimens possessing high viscosity, opacity, and columnar cell content are lower for the eugenol than for the clove oil. Since we have already cited reasons[3] for believing that *pure* gastric mucus is transparent, cell-free, and of variable consistency, it may be that the secretion yielded by eugenol differs less from pure mucus than does the fluid obtained with clove oil.

Five per cent eugenol emulsion is the most effective stimulus to mucus secretion which we have found to date—especially since it yields larger volumes of mucus which tend to have a higher pH than those induced by any of the other stimuli. The latter characteristic indicates also that eugenol has virtually no stimulating effect on the parietal cells. Furthermore, this substance is a pure compound, the major component of clove oil, whereas the latter is a mixture of at least 7 different chemical substances. Since the minor components of the essential oil may also exert some physiological effect on the mucosa, their absence from the pure eugenol enhances its value as a standard. Hence, we propose to adopt an aqueous emulsion of eugenol as a standard stimulating agent for further work on the physiology of gastric mucous secretion.

[6] Wood, H. C., Jr., and Osol, A., *The Dispensatory of the United States of America*, 23[e] ed., Philadelphia, 1943.

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The Influence of Coramine on the Liver of the Young Rat.

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Coramine (nikethamide) is a compound which provokes a number of different pharmacological actions. It is capable of curing black tongue in dogs by virtue of its close structural relationship to nicotinamide;[1] it is often employed as a medullary stimulant; and it has a pronounced ability to increase the liver weight of young rats.[2] Coulson and Brazda[2,3] have presented evidence which indicates that the unsubstituted nitrogen of the heterocylic ring and the di-ethyl substitution of the amido nitrogen are both in part responsible for the liver enlargement which follows the administration of the compound.

In preliminary experiments the absolute liver weight increase caused by coramine by the end of a 28-day period could not be prevented by the inclusion of 1.2% methionine or 0.5% choline in the diet.[2] This weight increase suggested that the liver cells were being injured by the coramine and that this was followed by the very rapid regeneration which is seen in many types of liver injury. It was deemed desirable to design experiments

[1] Smith, D. T., Margolis, G., and Margolis, L. H., *J. Pharm.*, 1940, 68, 458.

[2] Coulson, R. A., and Brazda, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 1.

[3] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, 62, 19.

Colormetric estimation of eugenol

in oil of clove, allspice, bay

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Some volatile oils which contain eugenol as the chief constituent are assayed according to their phenolic content by the alkali hydroxide method.[1] Guenther,[2] however, reported that any alkali-soluble or water-soluble constituents or adulterants will dissolve in the aqueous phase leading to an erroneous phenolic content. Some color tests have been discussed for the identification of eugenol. Kobert[3] stated that eugenol gives a red color with phloroglucinol and hydrochloric acid. Recently Wahba and Sinsheimer[4] stated that the red color with phloroglucinol and hydrochloric acid is due to coniferyl aldehyde, which is a common constituent of oils containing eugenol.

Folin-Denis reagent[5] and diazotized sulphonic acid[6] were applied to different phenols.

In their experiment the authors made use of Gibb's reaction[7,8,9] to assay eugenol in the presence of other accompanying phenols. The reaction is based on coupling with 2:6-dichloro- or dibromoquinone chloroimide in a veronal buffer. The authors made some modifications to suit the conditions required for the determination of eugenol. The blue color was found to develop best in a phosphate buffer instead of in veronal.

Materials

Eugenol was purified by distillation under nitrogen; purity was confirmed by physico-chemical methods. B.P. at 5 mm Hg 110-111°C; $d^{20} = 1.0664$; n, 1.5110.

Eugenol solution: 4 mg/100 ml isopropanol.

Oils containing eugenol: oil of clove (Antoine Chiris), white oil of clove (N.V. Chemische Fabrick, E. Landt), oil of bay (Edward Butiner), and oil of allspice (Edward Butiner).

Color reagent: dissolve 50 mg 2:6-dichloroquinone chloroimide (A.R.) in 100 ml of isopropanol, preparing the reagent only shortly before use.

Phosphate buffer: pH 8.6, prepared.

Experimental

The proposed method is based on the fact that eugenol can be estimated by measuring the intensity

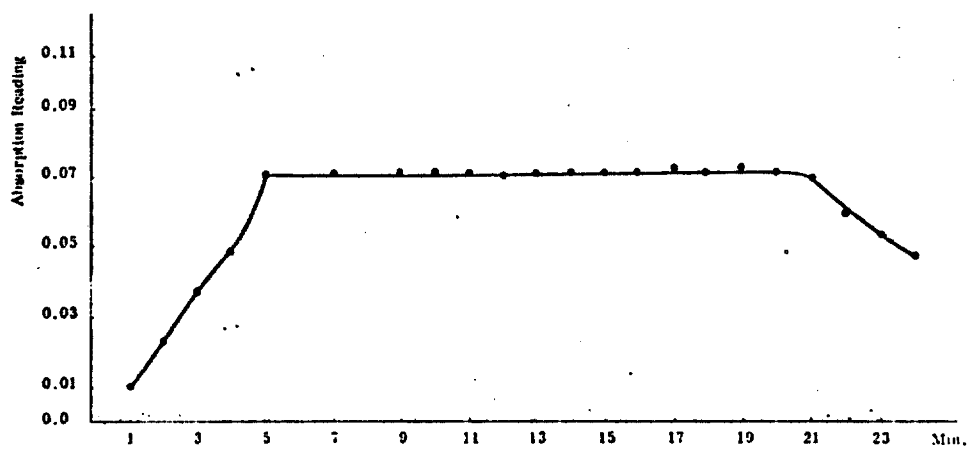

Fig. 1. Effect of time on the stability of color.

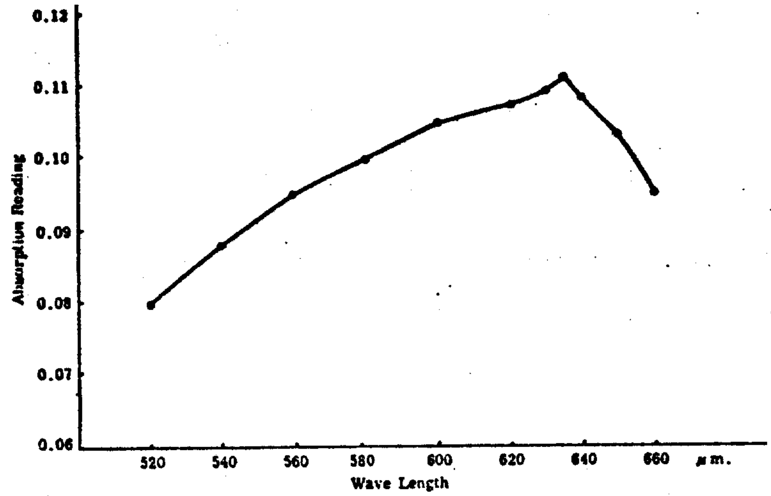

Fig. 2. Absorption spectrum of the color between 520 and 660 mµ.

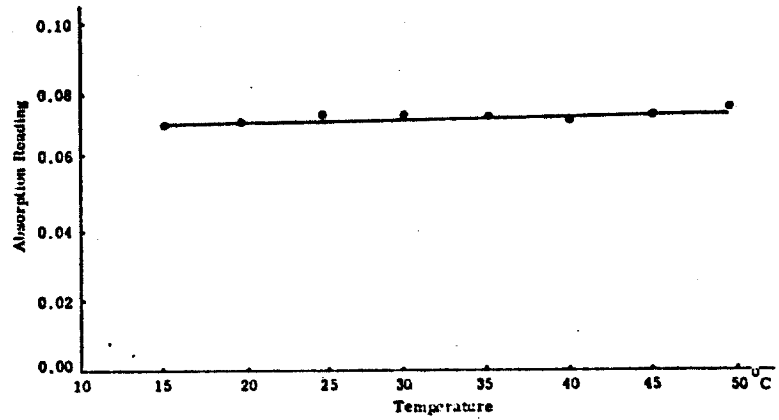

Fig. 3. Effect of temperature on the stability of color.

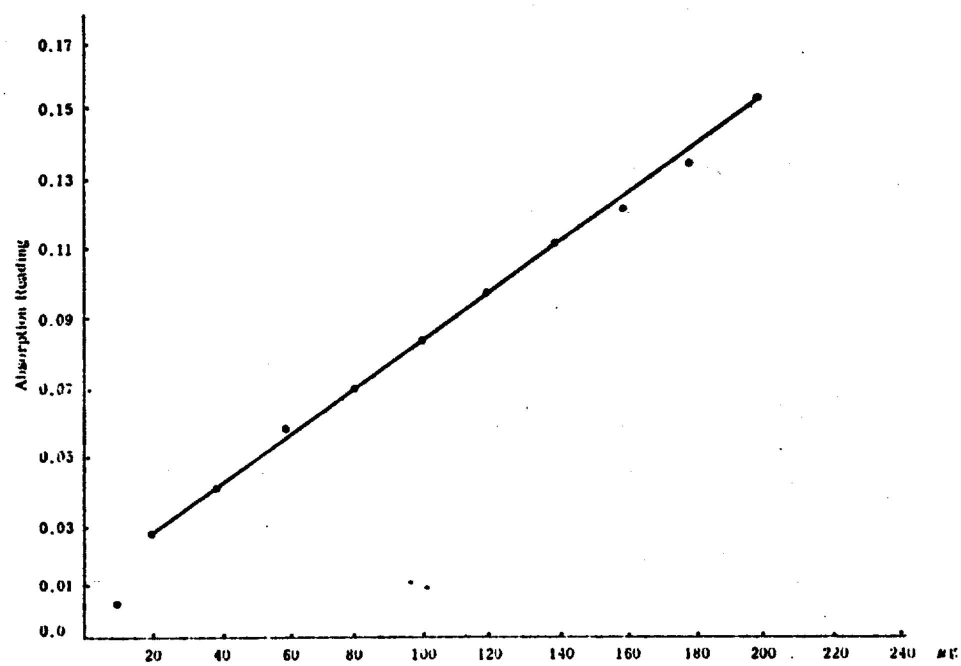

Fig. 4. Obedience of color to Beer's Law.

of the blue color developed by the reaction with Gibb's reagent in alkaline medium. Mix 1 ml of material with 5 ml isopropanol, 1 ml of color reagent, and 2 ml of the buffer solution. The different factors which might affect the stability and the sensitivity of the color were studied.

a) Effect of pH. Different buffers were tried, viz., veronal,[9] ammonium hydroxide-ammonium chloride,[10] a mixture of equal volumes of the ammonia buffer and 20 percent sodium acetate,[11] sodium acetate and phosphate buffers. A phosphate buffer of pH 8.6 was found to be the most suitable.

b) Effect of time. The stability of the color on standing at room temperature for different periods of time was studied. The color was measured immediately after the addition of the reagent and at one-minute intervals. The results are shown in Figure 1 which shows that the color reaches its maximum intensity after 5 minutes, remains stable for another 15 minutes and then begins to fade slowly.

c) Specific wave length. The color absorption was studied at different wave lengths in the visible spectra. The optimum wave length was found to be 635 mμ, using red photocell as shown in Figure 2.

d) Effect of temperature. The stability of the color at different temperatures was studied. The color was measured at temperatures ranging from 15° to 50° C. The results obtained are shown in Figure 3 which shows that the color is not affected much with the variation of temperature. Meanwhile, acetyl eugenol did not give any color when similarly treated.

e) Effect of concentration. The sensitivity of the

Table I. Analysis of solutions containing different amounts of eugenol.

Weight of eugenol added in μg	Reading	Weight of eugenol found in μg	Error %
40	0.045	40.0	0
80	0.072	80.0	0
100	0.085	99.4	-0.6
160	0.125	158.8	-0.75
200	0.155	202.6	+1.3

color at different concentrations was studied. Increasing aliquots of eugenol solution, ranging from 0.25 to 6.0 ml representing a concentration range of eugenol from 10 to 240 μg, were used. The aliquots were transferred to test tubes and each aliquot was completed to 6.0 ml with isopropanol. To each test tube exactly 1.0 ml of the color reagent was added, followed with 2.0 ml of the phosphate buffer (pH 8.6).

The test tubes were shaken gently for a short time to allow miscibility and hasten the color formation. A deep blue color developed in each test tube. The solutions were then transferred to the cuvettes of a Unicam S.P. 500 and the reading was recorded for each concentration at 635 mμ. A blank experiment was carried out under the same conditions using 6.0 ml of isopropanol instead of the eugenol solution.

An absorption concentration curve for pure eugenol was constructed, from which it is clear that the color obeys Beer's law in the range of 20–200 μg (Fig. 4).

The sensitivity and feasibility of the method were tested on isopropanol solutions containing known amounts of eugenol. The results are shown in Table I.

f) Effect of color reagent on other volatile oil con-

Table II. Effect of color reagent on some volatile oil constituents.

Constituents	Color	Constituents	Color
Eugenol	deep blue	Furfural	no color
Acetyl eugenol	no color	Cineole	no color
Isoeugenol	no color	- Pinene	no color
Chavicol	no color	Phellandrene	no color
Methyl Chavicol	no color	Caryophellene	no color
Vanillin	v.faint blue	Limonene	no color
Methyl Salicylate	no color		

stituents. Other phenolic and nonphenolic constituents which may normally occur with eugenol in volatile oils were tested by the same color reagent. The results obtained are shown in Table II.

From the Table II it is clear that the mentioned substances other than eugenol fail to give any persistent color. Vanillin produces an imperceptible blue color only in high concentrations. Hence it does not interfere as it occurs naturally in trace amounts in the oils containing eugenol.

Analysis of some essential oils containing eugenol

Different samples of oils (see under Materials) were placed in solution with isopropanol and their eugenol content was determined.

Procedure: In a test tube introduce 6.0 ml of an oil–isopropanol solution (20–200 μg eugenol) followed by 2 ml of phosphate buffer (pH 6.8) and 1 ml of the color reagent. Shake gently and set aside for 5 minutes.

Within the next 15 minutes measure the absorption of the blue color developed at 635 mμ, using a red photocell against a blank prepared instantaneously.

Deduce the percentage of eugenol from the absorption concentration standard curve of pure eugenol.

A comparison between the Egyptian Pharmacopoeia and the proposed colorimetric methods was made. The results are shown in Table III.

Table III. Determination of eugenol in oil of clove, oil of allspice, and oil of bay.

Oil sample	Experiment No.	Colorimetric method: Wt. of oil in μg	Colorimetric method: Eugenol percent w/w	E. P. method: Vol. of oil in ml	E. P. method: Eugenol percent v/v
Oil of clove	1	30	91	10	92
	2	40	90	10	96
	3	50	90	10	96
	4	100	92	10	95
	Mean		90.75		94.75
Oil of allspice	1	100	52	10	57
	2	150	54	10	60
	3	200	54	10	60
	4	200	54	10	62
	Mean		53.50		59.75
Oil of bay	1	100	32	10	57
	2	150	32	10	58
	3	200	34	10	58
	4	300	34	10	60
	Mean		33		58.25

Discussion and conclusion

A colorimetric method has been established for the determination of eugenol in small amounts of oil of clove and other oils containing it. The method is based on measuring the intensity of the blue color produced by the reaction of eugenol with 2:6 dichloroquinone chloroimide in isopropanol solution and phospate buffer at pH 8.6. Other phenolic and nonphenolic constituents occurring naturally with eugenol do not interfere in the color reaction. Concentrations of eugenol ranging from 20 to 200 μg were found to obey Beer's law. The method was applied to analyze oil of clove, allspice, and bay.

From the above work it is clear that the method is feasible and the results are reproducible, with an error ranging between −0.75 and +1.3 percent (Table I). The difference between the results of the suggested method and those of the Egyptian Pharmacopoeia method (oil of clove, 5%; oil of allspice, 6.66%; and oil of bay, 25.33%) is due to the presence of other phenolic and nonphenolic constitutents which are alkalisoluble and fail to give the color test of eugenol.

Moreover, the proposed colorimetric method will estimate as little as 30–300 μg of the oil containing eugenol, while the Egyptian Pharmacopoeia method requires 10 ml of it.

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Influence of Calcium and Magnesium on Eugenol-Induced Desquamation of Mucus Epithelium in Gastric Pouches*†

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In order to find a satisfactory topical stimulus for the study of the physiology of gastric mucus secretion, numerous agents have been investigated in this laboratory (9, 10, 11). Gentle massage, aqueous ether (saturated), 5 per cent aqueous clove oil emulsion, 50 per cent ethyl alcohol, distilled water, isotonic and hypertonic NaCl, and ½ to 5 per cent aqueous eugenol emulsions were all found to induce desquamation of the surface epithelium in addition to acting as mucigogues. The most effective of these mucus stimuli was 5 per cent eugenol, and this also produced huge amounts of desquamation. Since there is reason to believe that pure gastric mucus is cell-free (9), this associated phenomenon of decreased cellular cohesion is of interest in relation to the general problem of gastric mucus function, but particularly with those aspects of the problem concerned with the gastric mucous barrier as a protective mechanism against chemical and physical irritants. The latter considerations arise especially in any investigation which is concerned with an exogenous, topical agent as an etiological factor in adenocarcinoma of the stomach. Furthermore, the loosening of the cement substance which results in this diminished cohesion may well be related to the difference between normal and cancerous tissues, which makes for the invasive character of the latter.

Some of the attempts to discover a common denominator for such decreases in cellular cohesion have resolved themselves around the ionic constituents of the intercellular cement. Overton (14) stressed the importance of an easily dissociating calcium salt as the basis of the cohesive material which binds cellular membranes. A partial explanation for the decreased cohesiveness of malignant cells has been related to a calcium deficiency of the tissues by Brunschwig *et al.* (1) and by Coman (5). Further support for the concept that calcium plays a role in the maintenance of cellular cohesion has been fostered by the observations that calcium-free solutions decrease the cohesiveness of normal epithelial cells (6), capillary endothelium (3), ciliated gill cells of *Mytilus* (13), and blastomeres of developing sea urchins (8). Methylcholanthrene, which is known to decrease the cohesiveness of squamous epithelial cells, likewise decreases the calcium content of the mouse epidermis (2).

Since both calcium and magnesium are constituents of cell membranes (16), both ions may be involved in the maintenance of cellular cohesion in general. Some foundation for this hypothesis is rooted in the fact that the reunion of sponge cells does not occur in the absence of either ion (15), and recently Zeidman (18) reported that the absence of calcium or magnesium or both decreases the cohesiveness of human squamous epithelium.

The present work was designed to determine whether calcium, alone or in the presence of magnesium, will diminish or prevent the reduction in cohesiveness of the gastric epithelium induced by the mucigogue, eugenol.

PROCEDURE

The study was conducted on five Heidenhain pouch dogs, using the technique previously described (9). One per cent eugenol emulsion was made in distilled water, or in $CaCl_2$ and $MgCl_2$ solutions at the several concentrations indicated in Table 1. Tergitol-Penetrant (1/40 per cent) was used to stabilize the emulsions, as heretofore. Since an acid pH also decreases the cohesiveness of cells (3), all the eugenol emulsions were buffered with $NaHCO_3$ (about 0.1 per cent in final concentration). Determinations of pH of the emulsion before and after its application to the pouch revealed no significant deviation from neutrality in the course of an experiment. Following addition of the bicarbonate to the $CaCl_2$ solutions of highest concentrations, there was a slow precipitation of $CaCO_3$, but

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† A preliminary report of this work was presented before the American Physiological Society at its recent meeting in Detroit. An abstract was published in Fed. Proc., 8:76, 1949.

this was barely perceptible within the first 15 minutes. Hence, the salt-containing eugenol emulsion was not prepared until immediately before administration to the pouch. Each test experiment was preceded or followed on the same day by a control experiment with eugenol emulsion in distilled water.

Volume, viscosity, and opacity of half-hour samples of mucus were recorded over a period of 2 hours following each application of stimulus. Viscosity and opacity were evaluated on a scale from 1 to 5, using the standards described by Sober *et al.* (17). High viscosity and opacity are generally considered indicative of high columnar cell content, since these three factors are statistically correlated in significant degree (9). However, the presence of such cells was confirmed by microscopic examination of smears stained with toluidine blue. This was considered essential because coagulated mucin may also contribute to the opacity of mucus in considerable measure.

Our finding may be interpreted in any one of three ways: (1) the reaction between eugenol and the cement substance may involve calcium and magnesium in an irreversible (possibly non-ionic) manner, so that the presence of these cations at the surface of action of the desquamatory agent exercises no effect on this chemical process; or (2) this chemical reaction may be reversible, but eugenol is so powerful a desquamatory agent that its mass law effect cannot be offset by even the highest concentrations of these alkaline earth ions, which can be maintained at the surface of the mucosa under the conditions of these experiments; or (3) the desquamatory action of eugenol may be entirely independent of calcium and magnesium, *i.e.*, the mucigogue may act on some part of the cement substance which does not contain these elements. Apropos of the second of these possible interpretations, it should be noted that the concentration of eugenol used in these experiments was only 1 per cent, rather than 5 per cent as is being employed in most of our other studies with this mucigogue.

TABLE 1

CHARACTERISTICS OF GASTRIC MUCUS SECRETED IN RESPONSE TO 1 PER CENT AQUEOUS EUGENOL EMULSIONS CONTAINING ABOUT 0.1 PER CENT $NaHCO_3$ AND OTHER SALTS

Eugenol emulsion made in:	No. of expts.	No. of samples	Average viscosity	Average opacity	Average volume	Columnar Cell content
Dist. H_2O	12	45	3.4	2.8	5.6	Considerable
0.02% $CaCl_2$	5	18	3.1	3.1	5.6	"
0.04% $CaCl_2$	3	10	4.6	2.6	2.6	"
0.06% $CaCl_2$	2	8	3.8	2.4	5.3	"
0.01% $MgCl_2$, 0.02% $CaCl_2$	2	8	3.2	2.9	6.4	"
0.03% $MgCl_2$, 0.06% $CaCl_2$	2	8	4.4	3.4	4.1	"

RESULTS

At none of the ionic concentrations here employed did the salts significantly affect the viscosity, opacity, and columnar cell content of the mucus produced by topical application of eugenol emulsion. The mean viscosity and opacity values (Table 1) are not appreciably different from those for the control experiments, and the smears invariably demonstrate the presence of a high columnar cell content.

DISCUSSION

The desquamating action of 1 per cent aqueous eugenol emulsion is not prevented by adding calcium, alone or with magnesium, to the emulsion. This observation is in accord with the work of Zeidman (18) in that he was unable to reverse the decreased cohesiveness of human squamous epithelium, induced by a calcium-free solution, by restoring the ion to the medium. On the other hand, Chambers (4) found that the diminished cohesiveness of capillary endothelium, induced by a calcium-free perfusate, is reversed by changing to normal Ringer's solution.

The calcium deficiency found in malignant tissues, and which has been associated with their ability to invade adjacent tissues, may reflect some alteration of a calcium-binding complex in the cement substance (12). The chemical character of this complex is uncertain, but a protein structure has been suggested (12), and it may even be a lipoprotein (7). The desquamating ability of eugenol might be attributed to its lipid solvent ability, but as yet this does not seem feasible because hypertonic NaCl likewise produces desquamation in considerable degree (9). Although there is good reason to believe that mucigogue action is not necessarily accompanied by desquamatory action, the problem of how to stimulate large quantities of a cell-free gastric mucous secretion still remains unsolved.

SUMMARY

1. The object of these experiments was to determine whether calcium and magnesium can reduce eugenol-induced desquamation of gastric columnar epithelium. This was studied in dogs by the topical

application to Heidenhain pouches of 1 per cent buffered ($NaHCO_3$) eugenol emulsions containing these ions at several different concentration levels.

2. Such eugenol emulsions exercise the same mucigogue and desquamatory actions as do control emulsions containing none of the added electrolytes.

3. This finding fails to give support to the idea of a possible relation between the desquamatory action of a gastric mucigogue and the process of invasion by cancerous tissue.

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III. The Anti-anemia Action of Eugenol.

(On the anti-anemia action of the effective ingredients of some stomachic tablets.)

1) Action of Eugenol on the Blood Image at Normal Time.

Five (5) milligrams of eugenol per kilogram of weight was injected once into the muscles of a domesticated rabbit and the variations in the blood image was observed as the time passed. An observation of the experimental result shows that the variations in red blood corpuscles and hemoglobin were within the range of the so-called physiological variations even after the injections. The same could be stated about white blood corpuscles. Next, five milligrams of Eugenol per kilogram of body weight was injected continuously at the rate of one injection a day for a period of seven days and the variations in the regular blood image were observed.

In this case, too, the experimental result showed that only slight varitions were registered both in red blood corpuscles and hemoglobin. Even the number of white blood corpuscles did not change markedly.

In view of the aforementioned experimental result, it is considered that Eugenol had no effect whatsoever on the blood image of a normal domesticated rabbit.

2) Action of Eugenol on Phenylhydrazine Anemia

(1) In the case where phenylhydrazine and Eugenol are used together.

In this experiment, 0.02 grams of phenylhydrazine alone per kilogram of body weight was continuously injected once a day for a period of seven days and, in addition, this and five milligrams of Eugenol per kilogram of body weight were injected for a period of seven days and the observations were continued during the period.

An observation of the experiment revealed the fact that both red blood corpuscles and hemoglobin tended to become gradually reduced by the continued injections of phenylhydrazine. Even when the injections of Eugenol were carried out alongside, there was no change in the degree of the reduction, with the trend toward further reduction being continued.

Next, both red blood corpuscles and hemoglobin uniformly tended to increase as previously after the completion of the injections, the normal state having been restored in a period of 23 days.

(Insert Figure 8 on p. 261. a. Number of days. b. Hemoglobin. c. Red blood corpuscles. d. White blood corpuscles. e. 5,000,000. f. 4,000,000. g. 3,000,000. h. 2,000,000.)

(Insert Figure 9 on p. 261. a. Number of days. b. Hemoglobin. c. Red blood corpuscles. d. White blood corpuscles. e. 5,000,000. f. 4,000,000. g. 3,000,000. h. 2,000,000.)

Based on the aforementioned experimental results, it appears that Eugenol has no effect whatsoever upon phenylhydrazine anemia.

(ii) In the case where Eugenol was fed after Phenylhydrazine Treatment.

After injecting 0,002 grams of phenylhydrazine per kilogram of body weight once every day for a period of 14 days on a continuous basis, five milligrams of Eugenol per kilogram of body weight was continuously injected until the blood image was completely restored to normalcy.

An observation of the experimental process revealed the fact that both red blood corpuscles and hemoglobin were gradually reduced as in the previous case by the injection of phenylhydrazine and, at the time when the injections were completed, the total number of red blood corpuscles which counted in the range between 4,510,000 and 4,760,000 before the start of the injections were reduced to the range between 2,430,000 and 2,760,000. In the case of hemoglobin, 86 per cent was reduced to the range between 65 and 67 per cent.

Simultaneous with the termination of the phenylhydrazine injections, the continuous injection of fine (5) milligrams of Eugenol per kilogram of body weight was carried out. As a result, it was found that both red blood corpuscles and hemoglobin gradually increased and they returned to the normal levels within a period of 23 days.

When the above result is compared with the result obtained in a control experiment, it is seen that, even in the case of the control, the original state is more or less restored in a period of 23 days when the injection of phenylhydrazine is stopped. Thus, Eugenol has no effect whatsoever upon the rate of recovery.

3) Action of Eugenol upon Toluylenediamine Anemia

Zero point zero one (0.01) gram of toluylenediamine per kilogram of body weight was singly injected once a day for a period of 14 days on a continuous basis. Then, five milligrams of Eugenol per kilogram of body weight was injected once a day for a period of 12 days on a continuous basis along with toluylenediamine (0.02 grams per kilogram of body weight for a period of seven days and 0.03 grams for a period of five days).

An observation of the experimental result shows the fact that, during the first 14 days when the injections of toluylenediamine alone were carried out, both red blood corpuscles and hemoglobin were gradually reduced and, in the case of red blood corpuscles, the range between 4,330,000 and 4,370,000 before the initiation of the injections changed to the range between 2,860,000 and 3,290,000 at the completion of the injections. In the case of hemoglobin, the range between 81 per cent and 86 per cent changed to the range between 74 per cent and 76 per cent.

Next, toluylenediamine and Eugenol were fed simultaneously. In a short period of time, there was observed an increase, with hemoglobin tending to gradually increase at first. After the completion of the injections, red blood corpuscles were resotred to the original state within a period of 16 to 17 days. In the case of hemoglobin, the restoration to the original state was observed to be somewhat slower.

An observation of the aforementioned experimental result shows the fact the Eugenol clearly has the effect of preventing toluylenediamine anemia.

(Insert Figure 10 on p. 262. a. Number of days. b. Hemoglobin. c. Red blood corpuscles. d. White blood corpuscles. e. 5,000,000. f. 4,000,000. g. 3,000,000. h. 2,000,000.)

(ii) In the case where Eugenol was Fed after Toluylene diamine Treatment.

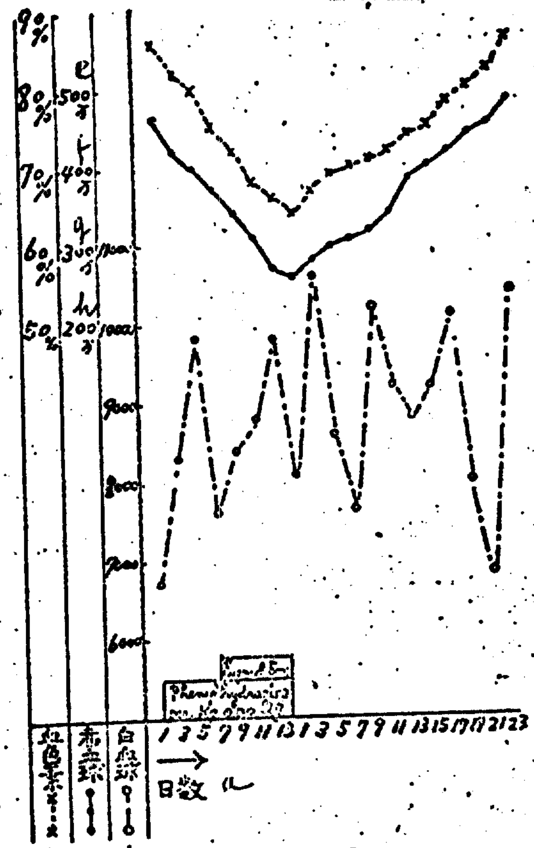
After continuously injecting 0.01 gram of toluylenediamine per kilogram of body weight for a period of 14 days, 0.02 grams of same per kilogram of body weight for a period of seven days and 0.03 grams of same per kilogram of body weight for a period of five days, the injection of Eugenol is an amount of five milligrams per kilogram of body weight was carried out on a continuous basis at the rate of one injection a day until restoration was achieved. Observations were carried out during this period of time.

An observation of the experimental result shows the fact that the continuous injections and toluylene diamine brought about a gradual decrease in red blood corpuscles and hemoglobin and, at the completion of the injections, the range of red blood corpuscles between 4,700,000 and 4,830,000 before the start of the experiment was reduced to 2,440,000. In the case of hemoglobin, the range between 86 and 87 per cent was reduced to 67 and 51 per cent.

Next, the injection of Eugenol was started upon the completion of the injection of toluylenediamine. There was a somewhat rapid increase in red blood corpuscles and hemoglobin and the normal state was completely restored within a period of 22 days.

A comparison of the aforementioned experimental result with the result obtained in the control shows that there seems to be a trend of some slight promoting effect, even though the difference observed was extremely small.

Translated by Carl Demrick Associates, Inc./RO/db



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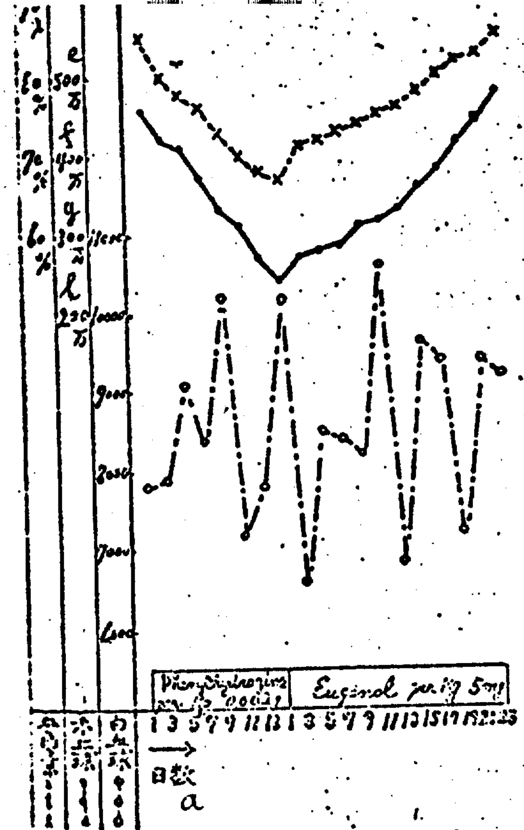

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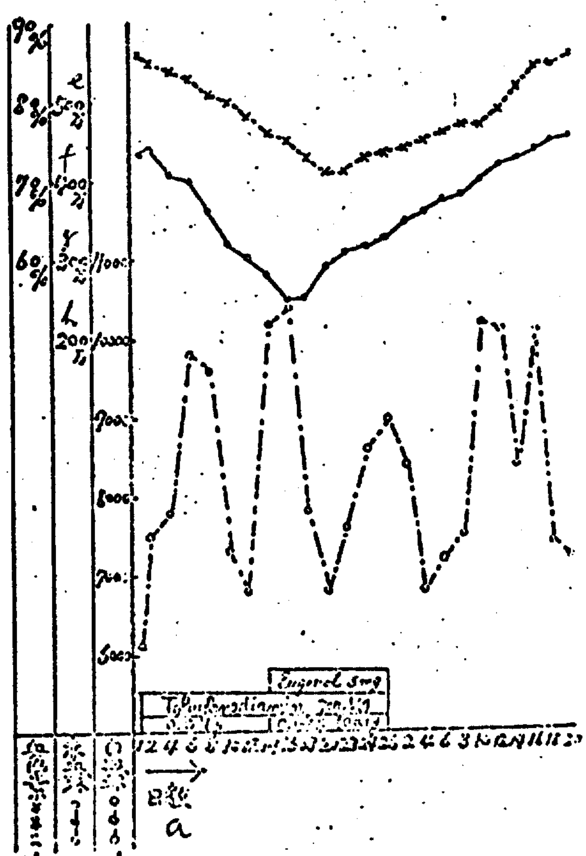

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Influence of Calcium and Magnesium on Eugenol-Induced Desquamation of Mucus Epithelium in Gastric Pouches*†

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In order to find a satisfactory topical stimulus for the study of the physiology of gastric mucus secretion, numerous agents have been investigated in this laboratory (9, 10, 11). Gentle massage, aqueous ether (saturated), 5 per cent aqueous clove oil emulsion, 50 per cent ethyl alcohol, distilled water, isotonic and hypertonic NaCl, and ½ to 5 per cent aqueous eugenol emulsions were all found to induce desquamation of the surface epithelium in addition to acting as mucigogues. The most effective of these mucus stimuli was 5 per cent eugenol, and this also produced huge amounts of desquamation. Since there is reason to believe that pure gastric mucus is cell-free (9), this associated phenomenon of decreased cellular cohesion is of interest in relation to the general problem of gastric mucus function, but particularly with those aspects of the problem concerned with the gastric mucous barrier as a protective mechanism against chemical and physical irritants. The latter considerations arise especially in any investigation which is concerned with an exogenous, topical agent as an etiological factor in adenocarcinoma of the stomach. Furthermore, the loosening of the cement substance which results in this diminished cohesion may well be related to the difference between normal and cancerous tissues, which makes for the invasive character of the latter.

Some of the attempts to discover a common denominator for such decreases in cellular cohesion have resolved themselves around the ionic constituents of the intercellular cement. Overton (14) stressed the importance of an easily dissociating calcium salt as the basis of the cohesive material which binds cellular membranes. A partial explanation for the decreased cohesiveness of malignant cells has been related to a calcium deficiency of the tissues by Brunschwig *et al.* (1) and by Coman (5). Further support for the concept that calcium plays a role in the maintenance of cellular cohesion has been fostered by the observations that calcium-free solutions decrease the cohesiveness of normal epithelial cells (6), capillary endothelium (3), ciliated gill cells of *Mytilus* (13), and blastomeres of developing sea urchins (8). Methylcholanthrene, which is known to decrease the cohesiveness of squamous epithelial cells, likewise decreases the calcium content of the mouse epidermis (2).

Since both calcium and magnesium are constituents of cell membranes (16), both ions may be involved in the maintenance of cellular cohesion in general. Some foundation for this hypothesis is rooted in the fact that the reunion of sponge cells does not occur in the absence of either ion (15), and recently Zeidman (18) reported that the absence of calcium or magnesium or both decreases the cohesiveness of human squamous epithelium.

The present work was designed to determine whether calcium, alone or in the presence of magnesium, will diminish or prevent the reduction in cohesiveness of the gastric epithelium induced by the mucigogue, eugenol.

PROCEDURE

The study was conducted on five Heidenhain pouch dogs, using the technique previously described (9). One per cent eugenol emulsion was made in distilled water, or in $CaCl_2$ and $MgCl_2$ solutions at the several concentrations indicated in Table 1. Tergitol-Penetrant (1/40 per cent) was used to stabilize the emulsions, as heretofore. Since an acid pH also decreases the cohesiveness of cells (3), all the eugenol emulsions were buffered with $NaHCO_3$ (about 0.1 per cent in final concentration). Determinations of pH of the emulsion before and after its application to the pouch revealed no significant deviation from neutrality in the course of an experiment. Following addition of the bicarbonate to the $CaCl_2$ solutions of highest concentrations, there was a slow precipitation of $CaCO_3$, but

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this was barely perceptible within the first 15 minutes. Hence, the salt-containing eugenol emulsion was not prepared until immediately before administration to the pouch. Each test experiment was preceded or followed on the same day by a control experiment with eugenol emulsion in distilled water.

Volume, viscosity, and opacity of half-hour samples of mucus were recorded over a period of 2 hours following each application of stimulus. Viscosity and opacity were evaluated on a scale from 1 to 5, using the standards described by Sober *et al.* (17). High viscosity and opacity are generally considered indicative of high columnar cell content, since these three factors are statistically correlated in significant degree (9). However, the presence of such cells was confirmed by microscopic examination of smears stained with toluidine blue. This was considered essential because coagulated mucin may also contribute to the opacity of mucus in considerable measure.

TABLE 1

CHARACTERISTICS OF GASTRIC MUCUS SECRETED IN RESPONSE TO 1 PER CENT AQUEOUS EUGENOL EMULSIONS CONTAINING ABOUT 0.1 PER CENT $NaHCO_3$ AND OTHER SALTS

Eugenol emulsion made in:	No. of expts.	No. of samples	Average viscosity	Average opacity	Average volume	Columnar Cell content
Dist. H_2O	12	45	3.4	2.8	5.6	Considerable
0.02% $CaCl_2$	5	18	3.1	3.1	5.6	"
0.04% $CaCl_2$	3	10	4.6	2.6	2.6	"
0.06% $CaCl_2$	2	8	3.8	2.4	5.3	"
0.01% $MgCl_2$, 0.02% $CaCl_2$	2	8	3.2	2.9	6.4	"
0.03% $MgCl_2$, 0.06% $CaCl_2$	2	8	4.4	3.4	4.1	"

RESULTS

At none of the ionic concentrations here employed did the salts significantly affect the viscosity, opacity, and columnar cell content of the mucus produced by topical application of eugenol emulsion. The mean viscosity and opacity values (Table 1) are not appreciably different from those for the control experiments, and the smears invariably demonstrate the presence of a high columnar cell content.

DISCUSSION

The desquamating action of 1 per cent aqueous eugenol emulsion is not prevented by adding calcium, alone or with magnesium, to the emulsion. This observation is in accord with the work of Zeidman (18) in that he was unable to reverse the decreased cohesiveness of human squamous epithelium, induced by a calcium-free solution, by restoring the ion to the medium. On the other hand, Chambers (4) found that the diminished cohesiveness of capillary endothelium, induced by a calcium-free perfusate, is reversed by changing to normal Ringer's solution.

Our finding may be interpreted in any one of three ways: (1) the reaction between eugenol and the cement substance may involve calcium and magnesium in an irreversible (possibly non-ionic) manner, so that the presence of these cations at the surface of action of the desquamatory agent exercises no effect on this chemical process; or (2) this chemical reaction may be reversible, but eugenol is so powerful a desquamatory agent that its mass law effect cannot be offset by even the highest concentrations of these alkaline earth ions, which can be maintained at the surface of the mucosa under the conditions of these experiments; or (3) the desquamatory action of eugenol may be entirely independent of calcium and magnesium, *i.e.*, the mucigogue may act on some part of the cement substance which does not contain these elements. Apropos of the second of these possible interpretations, it should be noted that the concentration of eugenol used in these experiments was only 1 per cent, rather than 5 per cent as is being employed in most of our other studies with this mucigogue.

The calcium deficiency found in malignant tissues, and which has been associated with their ability to invade adjacent tissues, may reflect some alteration of a calcium-binding complex in the cement substance (12). The chemical character of this complex is uncertain, but a protein structure has been suggested (12), and it may even be a lipoprotein (7). The desquamating ability of eugenol might be attributed to its lipid solvent ability, but as yet this does not seem feasible because hypertonic NaCl likewise produces desquamation in considerable degree (9). Although there is good reason to believe that mucigogue action is not necessarily accompanied by desquamatory action, the problem of how to stimulate large quantities of a cell-free gastric mucous secretion still remains unsolved.

SUMMARY

1. The object of these experiments was to determine whether calcium and magnesium can reduce eugenol induced desquamation of gastric columnar epithelium. This was studied in dogs by the topical

application to Heidenhain pouches of 1 per cent buffered ($NaHCO_3$) eugenol emulsions containing these ions at several different concentration levels.

2. Such eugenol emulsions exercise the same mucigogue and desquamatory actions as do control emulsions containing none of the added electrolytes.

3. This finding fails to give support to the idea of a possible relation between the desquamatory action of a gastric mucigogue and the process of invasion by cancerous tissue.

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TOXICITY OF THE MUCIGOGUE, EUGENOL, ADMINISTERED BY STOMACH TUBE TO DOGS

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INTRODUCTION

The secretory response of dogs' gastric mucosa to topical application of mild irritants has been under investigation in this laboratory[1, 2]. Eugenol (4-allyl-2-methoxyphenol) has been found to be a most effective stimulus for the secretion of highly viscous alkaline mucus, and has been adopted by us as a standard stimulus for investigation of the physiology of the gastric mucous barrier[3]. Previous work with this mucigogue was done with Heidenhain pouch dogs, but our current investigations required its administration to unoperated human beings[4] and dogs, and also to rodents. Since some of the stimulating agent may be transported into the small intestine and may not be recovered by aspiration, it became necessary to determine the tolerance and reactions of the animals to various doses of this compound. A review of the literature yielded only scanty information on the effects of this compound[5, 6, 7, 8].

EXPERIMENTAL PROCEDURE

Five healthy female mongrel dogs were employed for the 20 experiments of this study. They were all over 1 year of age and weighed about 10 kg. Food was withheld for 18–24 hours and water was removed from the cage about 15 hours (overnight) before the experiment. The dog was observed closely for one hour before administration of the stimulus, and pulse and respiration rates and temperature were determined at 30- or 60-minute intervals. An aqueous emulsion of eugenol (Merck, U. S. P.) was allowed to flow by gravity into the stomach, via a Levin tube.

The emulsion, stabilized with 5% gum acacia, was warmed to body temperature before administration. Emulsions of 2% and 5% concentrations of eugenol were administered in single doses of 50 or 100 ml. Following instillation of the mucigogue, observations were made on pulse, respiration, and temperature, as well as occurrence of retching and vomiting; character of stools; faltering gait; and general alertness, at least every half hour for 5–7 hours. Food and water were withheld throughout this experimental period.

RESULTS

Pulse and respiratory rate—Control (pre-treatment) pulse values were generally in the range of 84–140. In two experiments, however, the control values were 60–64, and in a third they were 144–156. There was frequently a difference of as much as 20 beats per minute in the three measurements taken at half-hourly intervals on the same day. The pulse rates taken during the 5-hour post-eugenol period were in essentially the same range (84—160) as the control values, although there were two particularly high values of 216 and 224. The mean for the 59 pre-treatment values was 103.6 ± 2.8 ($S.D._m$), whereas for 65 values following treatment, it was 117.7 ± 3.1—a difference which is significant at the 1% level of probability.

The observations on respiratory rates failed to show any definite effect of the eugenol, except in one dog (Exp. 267) during the pre-mortal period. The 53 control values were 12 to 50 (mean 26.5 ± 1.1), whereas the 55 experimental values varied from 10 to 36 (mean 23.8 ± 0.77). The difference between these two means is not significant at the 1% level of probability.

TABLE I

Summary of toxic effects following a single intragastric administration of eugenol emulsion in dogs

EUGENOL DOSAGE	NO. OF EXPS.	NO. OF DOGS	INCIDENCE OF REACTIONS (NO. OF EXPS.)		
			Vomiting	Ataxia	Death
100 cc.—5% (5.0 gm.)	6	4	4	3	2
50 cc.—5% (2.5 gm.)	7	4	2	None	None
100 cc.—2% (2 gm.)	6	3	None	None	None
50 cc.—2% (1 gm.)	1	1	None	None	None
Total..............................	20	5	6	3	2

Temperature—Rectal temperatures during the control periods ranged between 100.4 and 102.6°F. (59 values, mean 101.38° ± 0.02°). In the post-eugenol period, the mean temperature (N=66) fell slightly to 100.66° ± 0.06° and the range (98.8°—101.8°) reflected this small decrease also. The difference between these mean values (0.72°) is significant at the 1% level, in spite of the overlapping of the ranges, so that it may be inferred that the eugenol administration resulted in a slight depression of body temperature. However, examination of the data for individual experiments revealed no apparent correlation of temperature changes with volume or concentration of eugenol, nor with any other variable.

Systemic reactions—The highest dosage of eugenol in these tests was 100 ml. of a 5% emulsion. Severe reactions were noted in 5 of the 6 experiments (Table I), including 2 deaths within 24 hours after eugenol. In 3 of these experiments, retching and vomiting occurred between 1.5 and 4.5 hours after administration of the emulsion. In only one animal given this dosage was there no reaction whatever—a curious occurrence because in each of two subsequent experiments, with the same dosage run 6 or more days later, she manifested severe attacks of vomiting and retching. When the dosage was reduced by half (50 ml., 5% eugenol) there were no deaths. In only one of the 14 experiments at the low dosages was there any considerable vomiting, and in another, there was only a minor episode. At none of the dosage levels tested were there any signs of diarrhea or constipation.

In the case of the two dogs that died after receiving the maximal dose of eugenol, there was only slight vomiting in one of them. The second animal retained all 5 gm. of the toxic substance and was dead in 7 hours after its administration. In the case of the first dog, there was a slight reduction in quantity of eugenol absorbed, because of the loss by vomiting; this was evidently able to delay death by 24 hours, but not to prevent it. Survival of the dogs in 3 of the remaining experiments of this maximal dosage group can be ascribed to the extensive decrease in dosage which resulted from vomiting.

In 3 of the 6 experiments (3 dogs) employing the highest dosage (5 gm.), there occurred a motor dysfunction (ataxia) suggestive of a central nervous effect; such reactions were not noted at any of the lower dosage levels. In one of the dogs (Exp. 248), it was first observed at the end of the second hour following eugenol administration. On coaxing the animal to walk, it was noted that improperly coordinated hind leg movements, resulting in a "drunken" gait, persisted for about one-half hour. There had been a previous mild vomiting attack, but no recurrence noted thereafter. On the following morning, the dog was found dead. On autopsy, liver and kidneys were seen to be congested; stomach and duodenum had a mild hemorrhagic appearance. Histological examination of liver, stomach, duodenum, kidney, and adrenal gland revealed no additional abnormalities other than a mild venous congestion of the liver.

The second dog with motor disturbance (Exp. 267) first became ataxic about 3 hours after receiving 100 ml. of 5% eugenol. At this time, respiration was slow (about 10/min.), irregular, and deep; pulse rate was elevated to 180, and there was a subnormal temperature of 98.8° F. In this animal, the loss of coordination was seen in the front as well as hind limbs. After temporary improvement in gait, pulse rate, and temperature, the ataxia returned. At 6 hours, the animal lost consciousness, and died during the next hour. There was simultaneous cessation of respiratory and heart action. It is noteworthy that this dog gave no gross evidence of gastrointestinal distress throughout the experiment. Autopsy revealed a congested hyperemic liver, normal heart and kidneys, emphysematous lungs, contracted mottled spleen, and a slightly hyperemic pancreas. The corpus and fundus of the stomach appeared normal, as did the central portion of the antrum, but the remainder of the antrum and the pylorus were hyperemic with occasional small hemorrhages. The duodenal mucosa seemed normal, although there was much brown mucus present in this organ as well as in the stomach. Histologically, duodenum and stomach were normal. The kidney showed hyperemia of the glomeruli and parenchyma, with occasional cellular infiltration suggestive of a beginning glomerular nephritis. The liver showed marked venous congestion and early stages of necrosis of numerous cells, but no leucocytic infiltration.

Slight motor disturbances of the hind legs in the third dog (Exp. 275) were first noted 2 hours after eugenol was given. At this time the pulse rate was high (224/minute). There was considerable vomiting within the next half hour. By the end of the following hour (3.5–4 hours after eugenol), the ataxia had completely disappeared, and the dog was generally reactive and alert. No further signs of toxicity were observed following this recovery.

Throughout these toxicity studies, there was no indication that the eugenol exercised any effect on appetite. Following the 5–7 hour period of observation, the animals consumed moderate quantities of the food offered. Neither anorexia nor hyperorexia was observed thereafter.

Cumulative effects—Since the above experiments demonstrated that eugenol in a single 2 gm. dose had no immediate effect on dogs, 2 experiments were carried out in which 10 successive 2 gm. doses were administered at intervals of 48 to 72 hours. In general, the procedure was the same as before. The dogs acted normally after each administration; with no gastrointestinal, motor, or other systemic reactions noted at any time. Forty-eight hours after the last administration—on the twenty-third day—the animals were sacrificed. The autopsy and histological findings were essentially negative.

DISCUSSION

Of the several dosage levels at which eugenol was administered to dogs by stomach tube, only 5 gm./100 ml. of emulsion (5%) caused severe toxic symptoms— followed by death within 24 hours in 2 of the 6 experiments. However, when any considerable part of the material was expelled by vomiting, even as long as 2 hours after its administration, the animal was able to recover. It seems likely that the toxic effects operate at the level of the intestine rather than the stomach. This view is supported by two observations: First, by the failure of symptoms to develop before a substantial part of the eugenol has had time to pass down through the pylorus; and second, by the absence of systemic effects in our previous experiments with gastric pouch dogs in which as many as 7 fifteen-minute applications of 5% eugenol emulsions (usually 2.5 gm./dose) were made on a single day[9]. Independent of concentration or dosage, there was, in general, a depression of body temperature, confirming Mansfield[6] in this respect; but pulse rate was slightly increased and respiratory rate essentially unchanged.

That the substance in question is absorbed and has a marked effect on the nervous system may be inferred from the paralysis which starts in the lower extremities in both dogs and rats[10], and in some cases progresses to coma and even death. Organic effects are concentrated mainly in the liver, with congestion, hyperemia, and enlargement of cells predominating. The kidney is the site of similar mild damage in some cases. On the other hand, 20 gm. of eugenol administered to dogs in 10 divided doses over a period of about 3 weeks, induced no external manifestations of toxicity whatever. Such evidence of non-accumulation of the eugenol is in agreement with its rapid urinary excretion previously reported by Schroder and Vollmer[8].

For the dog, the highest dosage used that induced only minimal toxic effects was 2 gm./10 kg. or 0.2 gm./kg. Determination of LD50 in dogs is prohibitive because of cost and lack of uniformity of stock, but such a study has been carried out on adult albino rats[10]. When eugenol studies are performed on man or pouch dog, the material is allowed to remain in the stomach or pouch for only about 15 minutes, after which it is aspirated. Hence, the dosage levels found to be safe for orogastric administration in the present experiments (in which no aspiration was made) can be employed with a considerable margin of safety in clinical studies on human beings *with intact stomachs*. This does not apply to patients who have had a gastric resection or a gastroenterostomy, however, because of the great rapidity with which the eugenol may pass into their small intestines.

SUMMARY

A toxicity study is reported in which dogs were given intragastric instillations of eugenol emulsions, in order to determine the toxic reactions to this compound and the order of magnitude of safe dosage. In general, body temperature was slightly depressed, pulse rate increased, and respiratory rate was unaffected. Vomiting occurred only occasionally at a dosage level of about 2.5 gm./10 kg. body weight, but at the highest dosage employed (approximately 5 gm./10 kg.), its incidence was 65%. Marked motor dysfunction, primarily of the hind limbs, was observed only at this maximum dosage—in half of the experiments. Death occurred only after the highest dosage of eugenol in 2 of the 4 dogs used for the 6 experiments at this level. Thus, it would appear that a dosage of about 0.2 gm./kg. (100 ml. of 2% emulsion) is safe for experiments in dogs requiring orogastric administration of eugenol emulsion. Repeated administration at this dosage level (10 doses over a 3-week period) gave no indication of a cumulative effect.

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Percutaneous Resorption of Essential Oils and Their Ingredients

By Fr. Meyer and E. Meyer

It is widely held that essential oils can be absorbed percutaneously (v. Czetsch-Lindenwald and Schmidt-LaBaume, 1950, 1956). For a number of these substances the possibility of percutaneous resorption may be regarded as proven on the basis of the existing literature, but for many others exact experimental data are still missing.

Pfaffrath was able to show in 1934 that after percutaneous application essences of thyme, eucalyptus and turpentine are demonstrable in exhaled air and in some slight degree also in urine. Moncorps, Schmidt and Tholey (1937) were able to demonstrate guaiacol in urine after external use in the form of an ointment. Macht (1938) found that essential oils from cinnamon bark, fennel, betula, lemon, orange, anise, peppermint, thyme, Rosa geranium, Caryophyllus aromaticus, Gaultheria procumbens and others can cause, after percutaneous action of 1 cc, unconsciousness and lethal intoxication in mice. He successfully used some of them, e.g. anethole, as vehicles. Several alkaloids which as such or in cottonseed and olive oil are not absorbed percutaneously (among them morphine, strychnine, aconitine, atropine, eserine and curare) he was thus able to make effective. Bürgi (1942) jointly with Stähli also examined the resorption of essential oils and found that camphor, Oleum Eucalypti, Thymi, Citri, Terebinthinae, Rosmarini, Bergamottae, Pini, Juniperi and Lavendulae are detectable, after external application, in the exhaled air of rabbits by means of Bürgi's apparatus. In contrast to the studies of Macht (1938), in those of Bürgi (1942) oral and pulmonal absorption were certainly excluded; the treated skin portion was limited and the resorbing surface was thus constant. Recently Valette and Cavier (1945 a, b) made experiments about the resorption of essential oils and found particularly favorable values for alpha-pinene and eucalyptol. As eucalyptol is less volatile than alpha-pinene, it is especially recommended as vehicle. The authors referred to were able to cause for example dihydrofolliculin (1946 a), testosterone (1946 b), desoxycorticosterone (1947 a), synthetic estrogens (1948 a) and progesterone (194 b) in a mixture of ethanol and eucalyptol to be absorbed percutaneously in relatively large quantity.

Supplementing the findings of Valette and his school, we have included in our comparative studies on the permeability of the skin the essential oils accessible to us and their ingredients and have found great differences in the rate of resorption, as expected.

Some of the tested essential oils came from a collection of our Institute more than 10 years old. It is possible, therefore, that due to polymerization, for instance, the viscosity of some substances was higher than in fresh material and the rate of resorption was therefore found too low.

Method

If a liquid is resorbed by the skin, it functions also as vehicle. A compound incorporated or dissolved therein is absorbed together with it and thus can indic ate that resorption has taken place. Eserine is especially suitable as such an indicator (R. Vogel, 1899; G. Valette and R. Cavier, 1951) because it has characteristic, easily recordable effects on striated muscle. The latency between application on the skin and occurrence of the eserine effect on the periodically stimulated chewing muscles of mice was used as a measure of the rate of resorptio The contact or resorbing surfaces were 2.2 cm^2 of shaved abdominal skin of 250 male animals. The eserine concentration was 0.25% referred to the base. (For further details of the method cf. Meyer & Kerk, 1959).

Results

In Table 1 are shown some results with aliphatic essential oils and their constituents. In this first, chemically closely related group of compounds with 10-14 C atoms, geranyl formate was absorbed fastest (34 min). Then followed geranyl propionate (38 min), linalyl acetate (52 min), geranyl butyrate (55 min), geranyl acetate (58 min) and citral (63 min). Geraniol and linalool were not re sorbed within 2 hours in perceptible quantity. However, after subcutaneous injection of 10 gamma of eserine, dissolved in geraniol, there was no increase in the response, so that inactivation of the eserine (incompatibility) or an antagonistic influence on the eserine effect could not be ruled out with certainty. Fo linalool the tertiary OH group seemed to have an adverse effect on the resorption

Table 1

Vehicle for eserine (0.25% solution)	Min. to start of rise (arithmetic mean)	Number of tests evaluated
Citral	63	5
Geraniol	negative	4
Geranyl acetate, fresh	58	5
Geranyl acetate, viscous	negative	6
Gernayl formate	34	6
Geranyl propionate	38	5
Gernayl butyrate	55	5
Linalool	negative	5
Linalyl acetate	52	7
Citronellal, viscous	negative	6
Ol. Rutae	27	6
Valeric acid diethylamide	negative	3
Menthyl valerianate	43	5

That the viscosity plays an important part in percutaneous resorption is evident from the fact that we tested with negative result an old, thick geranyl acetate, while a fresh, fluid charge was resorbed already after 58 minutes in perceptible quantity. Also the test with viscous citronellal was negative.

Oleum Rutae, whose main constituent is methylnonyl ketone and which therefore belongs in the aliphatic series, is resorbed very well. Under the described test conditions we found a mean of 27 min. While during the test period limited to 2 h valeric acid diethylamide was not resorbed in perceptible quantity (3 animals), menthyl valerianate was evidently absorbed relatively quickly despite its great chain length.

Table 2 summariezes the result of the testing of alicyclic compounds, terpenes and those essential oils whose main constituent can be classified in these groups With the exception of two substances, solid terpin hydrate and an old, viscous grade of terpineol (terpinolene), all compounds of these groups were absorbed, in part after a surprisingly short time.

Table 2

Vehicle for eserine (0.25% solution)	Min. to start of rise (arithmetic mean)	Number of tests evaluated
Terpin hydrate 5% in cyclohexane	16	6
Terpin hydrate 10% in propanol	negative	6
Terpineol (*)	33	5
Terpineol, viscous	negative	7
Terpinyl acetate	50	6
Limonene	43	6
Carvone	35	5
Thymene = 1-pinene	22	6
Fenchone	45	6
Fenchyl acetate	54	5
Bornyl acetate	65	6
01. camphoricum	39	6
01. Pini sibirici	55	5
01. Terebinthinae	62	5
01. Tanaceti	38	5
01. Sabinae	48	5
01. Mentae puleg.	29	6
01. Eucalypti	31	5
01. Menthae pip.	58	5

(*) Fresh commercial product

The resorption of a 5% terpin hydrate solution in cyclohexane was recognizable 16 minutes after external application. It took place practically at the same speed as cyclohexane alone (cf. Meyer, Meyer & Kerk, 1959). A 10% solution of this substance in propanol, however, was not absorbed. Its addition, therefore, does not accelerate the resorption of propanol (cf. Meyer & Kerk, 1959). This means that with respect to percutaneous resorption terpin hydrate is rather indifferent.

Terpineol was absorbed relatively quickly (33 min), despite its tertiary OH group. Its esterification with acetic acid made the resorption slower instead of faster, as might have been expected from the described findings obtained with linalool and linalyl acetate (cf. Table 1). The mean calculated for terpinyl acetate was 50 min.

Limonene (43 min) and carvone (35 min), which occurs abundantly in caraway and dill oil, were resorbed fairly quickly.

In the terpenes and the terpene-containing essential oils, a dependence of percutaneous resorption on the chemical constitution was not seen.

Thymene = l-pinene was resorbed particularly well in this series (22 min). The followed fenchone (45 min), fenchyl acetate (54 min) and bornyl acetate (65 min). Despite a close chemical relationship, bornyl acetate and camphor (light camphor oil) were absorbed at greatly different speed (39 and 65 min).

Ol. Pini sibirici, which contains predominantly bornyl acetate, l-pinene and santene, as well as Ol. Terebinthinae with a relatively high alpha-pinene conten were resorbed but slowly. The mean values of 5 tests each were 55 and 62 min.

For Ol. Tanaceti with its high thujone content and for Ol. Sabinae which contains compounds chemically very similar thereto (sabinol and sabinol acetate) we found somewhat more favorable values: 38 and 48 min.

The rapid resorption of Ol. Menthae pulegiae (29 min) seems to be attributable to the 80% of pulegone contained therein. Also the menthone and menthol further contained in pulegol, as well as l-limonene and dipentene, are resorbable percutaneously. But their relatively small proportion suggests that they are of mino importance. Ol. Eucalypti contains, in addition to eucalyptene (alpha-pinene), pinocarveol, butyric, valeric and caprioic acid aldehyde, predominantly (ca. 75%) eucalyptol, the rapid cutaneous absorption of which Valette & Cavier (1945) had found before us. (Concerning local tolerance cf. Oettel. 1936).

The percutaneous resorbability of Ol. Menthae piperitae, which contains 30-50% menthol in addition to menthone, various menthone esters and terpenes, is comparable with Ol. Pini sibirici or Ol. Terebinthinae.

Table 3

Vehicle for eserine (0.25% solution)	Min. to start of rise (arithmetic mean)	Number of tests evaluated
Carvacrol	negative	6
Anethole	"	6
Ol. Anisi	"	5
Eugenol	"	6
Iso-eugenol	"	6
Safrol	72	6
Cumin oil	28	5
Cumin alcohol	87	5
Cumin aldehyde	47	6
Ol. Thymi	82	5
Ol. Petroselini	68	6
Cinnemal = Cinnamon aldehyde	negative	3
Cinnamein = Cinnamic acid benzyl ether	"	5

As can be seen from Table 3, in which are listed the aromatic ingredients of essential oils, carvacrol, anethole, Ol. Anisi, eugenol and iso-eugenol are not resorbed by the skin, or only very slowly so. For safrol, instead, the percutaneous absorption was clearly demonstrable within 2 h (mean value 72 min; concerni the different local tolerance cf. Oettel, 1936). Surprising is the short latenc period for cumin oil (28 min), which consists of p-cymol, little alpha- and beta-pinene, dipentene, beta-phellandrene, much cuminaldehyde and cumin alcohol. Since cumin alcohol and cuminaldehyde (mean values of 87 and 47 min) are not absorbed particularly quickly, it seems that p-cymol is primarily responsible for the resorbability of cumin oil. Ol. Petroselini (68 min) and Ol. Thymi (82 min) were resorbed relatively slowly, cinnameine and the very viscous cinnamon aldehyd not in perceptible quantity.

Some essential oils which cannot readily be classified in the groups described until now because of their ingredients are listed in Table 4.

Table 4

Vehicle for eserine (0.25% solution)	Min. to start of rise (arithmetic mean)	Number of tests evaluated
Ol. Calami	negative	5
Pepper oil	38	5
Ol. Galangae	33	6
Patchouli oil	negative	4
Ol. Fagi ether.	"	6
Tolubalsam oil	"	4
Ol. Copaivae	92	5
Ol. Pimentae	negative	6
Ol. Origani cretici	"	6
Ajowan oil	"	6
Ol. Spicae	"	5
Ol. Juniperi	59	6

Except for Ol. Galangae and pepper oil, they are resorbed much more slowly than the oils named in Tables 1 to 3. The values found are 1 hour (Ol. Juniperi 59 min) or much more (Ol. Balsam. Capaiv. 92 min). For Ol. Calami, Fagi aether., Pimentae, Origani cretici and Spicae as well as for patchouli, tolubalsam and ajowan oil, resorption is not detectable during the observation period of 2 h.

To test the percutaneous toxicity, the relatively well resorbing compounds fro Tables 1 to 4 were applied under the same conditions - but without eserine addition - on the skin of 2 mice each, for 4 hours. The result of these orientative control tests is summarized in Table 5.

It shows that essential oils in contact with the epidermis are not indifferen

A dependence of the percutaneous toxicity on the rate of resorption was, however, not ascertainable.

Table 5

Vehicle	Body weight in g		Exitus letalis after h		Finding after 4 h	
Citral	15	16	½	1½	-	-
Geranyl acetate	16	16	4	-	-	no f.
Geranyl formate	12	19	-	-	no f.	no f.
Geranyl propionate	19	18	-	4	no f.	-
Geranyl butyrate	26	19	-	-	no f.	no f.
Linalyl acetate	13	17	-	2	no f.	-
01. Rutae	25	25	4	-	-	no f.
Menthyl valerianate	18	26	3	-	-	no f.
Terpineol	12	18	2½	-	-	no f.
Terpinyl acetate	16	19	-	-	no f.	no f.
Limonene	20	14	-	-	no f.	no f.
Carvone	18	21	-	-	no f.	no f.
Thymene	21	17	-	3½	no f.	-
Fenchone	18	19	-	-	no f.	no f.
Fenchyl acetate	18	24	-	-	no f.	no f.
Bornyl acetate	22	20	-	-	no f.	no f.
01. camphoricum	21	26	-	-	no f.	no f.
01. Menthae pulegiae	17	16	-	-	no f.	no f.
01. Tanaceti	16	17	-	4	no f.	-
01. Sabinae	17	19	-	-	no f.	no f.
01. Eucalypti	19	16	-	2	no f.	-
01. Pini sibirici	17	11	-	-	no f.	no f.
01. Terebinthinae	18	25	-	1½	no f.	-
Safrol	24	19	3	-	-	no f.
Cumin oil	21	13	3	-	-	no f.

Table 5 continued.

Cumin alcohol	23	19	-	4	no f.	-
Cumin aldehyde	20	20	-	-	no f.	no f.
Ol. Petroselini	24	16	-	-	no.f.	no f.
Pepper oil	15	19	-	-	no f.	no f.
Ol. Galangae	26	22	-	2	no f.	-

Summary

(given in English in original publication, p. 519)

Literature

(requires no translation, see p. 519)

Translated by Carl Demrick Associates, Inc./IH/db

(132a)

ml with 10% alcohol (1, 2, 4, 6, 8, 10 ppm benzaldehyde).

19.097 ***Determination***

Pipet sample (usually ca 5 ml flavor or 25 ml cordial) into distn flask. Add enough alcohol to ensure min. of 10% alcohol in distillate. Add ca 110 ml H_2O to flavor or 200 ml H_2O to cordial and distill, collecting 100 ml or 200 ml, resp. If necessary, dil. aliquot of distillate with 10% alcohol to produce *A* of ca 0.5 at 249 nm, using 10% alcohol blank.

Det. *A* of std benzaldehyde solns at 249 nm against blank of 10% alcohol, and plot std curve.

Det. benzaldehyde concn from *A* of sample at 249 nm and std curve, or calc. av. *A* of 1 ppm benzaldehyde (A'). Concn of benzaldehyde in ppm = $(A/A') \times F$, where F is diln factor. (For most accurate work conduct 5 ppm std with each detn.)

For flavors giving higher *A* than std at 222 nm, subtract av. of *A* for min. at 222 and 350 nm from *A* for max. at 249 nm to calc. *A*.

19.098 Benzoic Acid (36)—Official First Action

Measure 10 ml sample into 100 ml flask and add 10 ml 10% NaOH soln and 20 ml 3% H_2O_2 soln; cover with watch glass and place in 100° oven. Oxidation of aldehyde to benzoic acid begins almost immediately; continue heating 5–10 min after all benzaldehyde odor disappears (20–30 min).

Remove flask from oven; transfer contents to separator, rinsing off watch glass; add 10 ml H_2SO_4 (1 + 5); and cool contents of funnel to room temp. under tap. Ext benzoic acid with 25, 25, 20, and 20 ml portions ether, and wash combined exts with 2 portions of 5–10 ml H_2O, or until all H_2SO_4 is removed. Filter into weighed dish, evap. at room temp., dry overnight in desiccator, and weigh the benzoic acid. Multiply result by 10.

Multiply g/100 ml benzaldehyde obtained in 19.093, 19.095, or 19.097 by 1.151 to obtain equiv. of benzoic acid and subtract this product from g/100 ml total benzoic acid obtained above. Difference = g benzoic acid/100 ml ext.

Hydrocyanic Acid

19.099 ***Qualitative Test—Procedure***

To several ml sample add several drops freshly prepd 3% $FeSO_4.7H_2O$ soln and single drop 1% $FeCl_3.6H_2O$ soln. Mix thoroly and add 10% NaOH soln, dropwise, until no further ppt forms and then H_2SO_4 (1 + 9) to dissolve ppt. In presence of even small quantities of HCN, Prussian blue coloration or suspension develops.

19.100 ***Quantitative Method—Official Final Action***

(In absence of chlorides)

Measure 25 ml sample into small flask and add 5 ml *freshly pptd* $Mg(OH)_2$, Cl-free. Titr. with 0.1*N* $AgNO_3$, using K_2CrO_4 as indicator. 1 ml 0.1*N* $AgNO_3$ = 0.0027 g HCN.

Nitrobenzene

19.101 ★ ***Qualitative Test—Procedure*** ★

See 19.094, 10th ed.

CASSIA, CINNAMON, AND CLOVE EXTRACTS

Alcohol—Official Final Action

19.102 ***Method I***

See 19.091.

19.103 ***Method II (33)***

Det. sp gr of ext at 15.56/15.56° or 20/20° as in 9.011, and oil as in 19.105, and apply formula given in 19.050. Use following values for sp gr of the oil: cassia, 1.05; cinnamon, 1.03; and clove, 1.055.

19.104 Isopropanol—Official Final Action

Proceed as in 19.052–19.054.

19.105 Oil (37)—Official First Action

Pipet 10 ml sample into Babcock milk test bottle. Remove nearly all alcohol by blowing air into bottle thru small glass tube 30 min, or longer if necessary. From 10 ml buret add 1 ml *solv.* (equal parts USP mineral oil and H_2O-free kerosene), shake well, and fill with satd $MgSO_4$ soln. Centrf. 10 min and read vol. of oil from extreme bottom to extreme top of column. To obtain % oil subtract 5 divisions and multiply remainder by 2.

GINGER EXTRACT

19.106 Alcohol—Official First Action—*See* 9.013

19.107 Solids (38)—Official First Action

Evap. 10 ml sample nearly to dryness on steam bath, dry 2 hr in oven at temp. of boiling H_2O, and weigh.

19.108 Ginger (Qualitative Test) (39)—Official First Action

(*Caution: See* 46.011, 46.039, and 46.054.)

Dil. 10 ml sample to 30 ml, evap. to 20 ml, decant into separator, and ext with equal vol. ether. Let ether evap. spontaneously in porcelain dish, and to residue add 5 ml 75% H_2SO_4 (by wt) and ca 5 mg *vanillin*. Let stand 15 min and add equal vol. H_2O. In presence of ginger ext, soln turns azure blue.

19.109 ★ **Capsicum (Qualitative Test) (40)—Official First Action** ★

(*Caution: See* 46.011, 46.039, and 46.054.)

See 19.102, 10th ed.

Vanillin

19.008 ***Ultraviolet Screening Method (2)—Official Final Action***

(*Caution:* See 46.016.)

(In absence of coumarin and Et vanillin)

Pipet 5 ml sample (for imitations and concentrates, use 2 ml) into 100 ml vol. flask, dil. to vol. with H_2O, and mix well. Pipet 2 ml of this soln into second 100 ml vol. flask, add 2 ml 0.1*N* NaOH, and dil. to vol. with H_2O. Pour ca 20 ml of this soln into small beaker and place under UV lamp in dark room. If coumarin is present to extent of 0.01% in original ext, brilliant green fluorescence will develop in 5 min.

If no coumarin is observed, read *A* of remaining alk. soln at 270, 348, and 380 nm. Obtain background $A = 0.29 \times A_{270} + 0.71 \times A_{380}$. Subtract this value from A_{348} and divide by *A* of 1 ppm vanillin (ca 0.150), detd from std soln of 3 ppm vanillin contg 2 ml 0.1*N* NaOH in 100 ml, and multiply by diln (1000) to give ppm vanillin in original sample. Make all readings within 2 hr of final diln.

If background *A* is too high (>0.15), clarify with isopropanol as follows:

Pipet 5 ml sample into 50 ml vol. flask and dil. to vol. with isopropanol. Transfer to centrf. bottle and centrf. ca 10 min at high speed. Without disturbing sepd solids, carefully pipet 1 ml liq. into 100 ml vol. flask, add 2 ml 0.1*N* NaOH, dil. to vol. with H_2O, and proceed as above.

Prep. std vanillin soln by dissolving 0.1000 g vanillin in 3 ml alcohol in 100 ml vol. flask, and dil. to vol. with H_2O (1 ml = 1 mg). Pipet 3 ml into 1 L vol. flask, add 2 ml 0.1*N* NaOH, and dil. to vol. with H_2O. Det. *A* at 270, 348, and 380 nm against H_2O contg 2 ml 0.1*N* NaOH dild to 100 ml with H_2O. Calc. corrected *A* as $A_{348} - (0.29A_{270} + 0.71A_{380})$, where A_{348}, A_{270}, and A_{380} are observed *A* at these wavelengths. Divide this value by 3 to obtain corrected *A* of 1 ppm vanillin.

Ultraviolet Spectrophotometric Method (3)—Official Final Action

19.009 ***Preparation of Standard Curve***

Dissolve 0.100 g vanillin in 5 ml alcohol and dil. to 100 ml with H_2O. Transfer 15, 10, and 5 ml, resp., to 250 ml vol. flasks, dil. to vol. with H_2O, and mix (*Solns A*). Pipet 10 ml of each *Soln A* into 100 ml vol. flask, dil. to vol. with H_2O, and mix. Pipet another set of 10 ml *Solns A* into 100 ml vol. flasks, add ca 80 ml H_2O and 2 ml 0.1*N* NaOH, mix, dil. to vol. with H_2O, and mix again. Obtain *A* of alk. solns at 348 nm, using neut. solns as ref. blanks. Plot std curve.

19.010 ***Determination***

If sample contains >0.3 g vanillin/100 ml, dil. with 35% alcohol to below this level. Pipet 10 ml sample (or dild sample) into 100 ml vol. flask, dil. to vol. with H_2O, and mix. Pipet 2 ml dild soln into each of two 100 ml vol. flasks. Dil. one with H_2O and mix. To other flask add 80 ml H_2O and 2 ml 0.1*N* NaOH, mix, dil. to vol. with H_2O, and mix again. Det. *A* of alk. soln at 348 nm, using neut. soln as ref. blank. Obtain vanillin content from std curve.

Vanillin and Ethyl Vanillin (4)—Official Final Action

19.011 ***Reagents and Apparatus***

(a) *Mobile solvent.*—Cyclohexane (practical)-EtOAc-MeOH, 100 + 30 + 20.

(b) *Immobile solvent.*—10% dimethylformamide in ether.

(c) *Sodium carbonate soln.*—Dissolve 4 g Na_2CO_3 in H_2O and dil. to 1 L.

(d) *Chromatographic paper.*—Whatman No. 3 MM, 8 × 8".

(e) *Chromatographic tank.*—Mitchell tank and equipment, 29.007(a).

(f) *Spotting pipet.*—10 µl.

(g) *Long wave ultraviolet light.*—(*Caution:* See 46.016.)

19.012 ***Preparation of Standard Curve***

Prep. solns of vanillin and Et vanillin in 35% alcohol, contg 0.10, 0.15, 0.20, 0.30, and 0.40 g/100 ml. Draw parallel lines on chromatgc paper 1" and 1½" above bottom edge, using hard pencil. Apply one 10 µl spot of each soln on the 1" line, keeping spots 1" apart and starting 2" from left side of paper. Use sep. papers for vanillin and Et vanillin curves. Use same micropipet for all spottings, rinsing thoroly before each application. Let spots air-dry, without heat. Handle paper carefully near edges to avoid high blanks.

Meanwhile place 100 ml H_2O in bottom of chromatgc tank contg one trough. Fill trough with mobile solv., cover tank, and seal. Let stand 15 min. Dip paper into immobile solv. from top down to 1½" line, leaving bottom 1½" of paper free from immobile solv. Do not permit solv. to reach spots. (Dipping can readily be done by use of shallow pan contg solv.) Air-dry paper few min, remove seal from tank, and place paper in tank with bottom edge dipping into mobile solv. Reseal tank and develop 2 hr, even tho solv. front reaches top before end of this period. Remove paper and air-dry. Do not expose developed paper to air >1 hr. If delay is necessary, place paper in jar and store in refrigerator.

Expose paper to NH_3 fumes for few min by placing paper in wide-mouth half-gal. jar contg small beaker with NH_3 on bottom, and capping jar. Examine paper under long wave UV light and outline dark blue areas with soft pencil. Et vanillin will show higher R_f value than vanillin. Remove marked areas with scissors and cut each into smaller pieces before placing them in 50 ml erlenmeyers. Cut out 2 blanks from side of paper, each approx. equal in area to developed spots. Use side area for these blanks, away from spotted areas and their developed rise.

Pipet 10 ml Na_2CO_3 soln into each flask, swirl, and let stand 10–15 min, with frequent swirling. Centrf. or filter thru rapid paper, discarding first portion of filtrate. Det. A at 348 nm, using Na_2CO_3 soln as ref. Also obtain av. A of the 2 blanks and correct std A before plotting std curve.

19.013 ***Determination***

If sample contains >0.4 g vanillin/100 ml, dil. below this level with 35% alcohol. Make one 10 μl spotting on the 1″ line with same micropipet used to prep. std curves. Proceed as above and det. vanillin and Et vanillin by comparison with appropriate std curves.

Coumarin—Official Final Action

19.014 ★ ***Photometric Method (5)*** ★

See 19.009–19.011, 10th ed. (*Caution: See* 46.008.)

Vanillin, Ethyl Vanillin, and Coumarin (6)—Official Final Action

Chromatographic Separation Method

19.015 ***Apparatus***

(**a**) *Spectrophotometer.*—Capable of detg A at 270 and 325 nm. Adjust to high sensitivity to utilize slit width <10 nm.

(**b**) *Silica cells.*—1 cm. Match cells at 270 and 325 nm, using isooctane-$CHCl_3$ solv., 19.016(c). (This solv. dets differences that other media do not.) Cells must be free of other solvs before adding isooctane-$CHCl_3$ solv., std, and sample solns. Drain each cell well between readings by inverting on towel. Fill cells for reading so that meniscus is >3 mm above light path.

(**c**) *Chromatographic tube.*—Melt glass tube 11–12.5 mm id ca 18″ from one end, draw out short distance, cool, and break at constriction. Partially close constricted end in flame, and dry.

19.016 ***Reagents***

(Same batch of isooctane must be used to prep. solv. mixt. and all dilns for set of detns.)

(**a**) *Silicic acid.*—Reagent grade "100-mesh" powder, suitable for chromatgy (Mallinckrodt Chemical Works No. 2847, or equiv.). Det. SiO_2 content as follows: Accurately weigh ca 1 g silicic acid into weighed Pt crucible. Ignite in muffle 15 min at 615°, cool in desiccator, and reweigh. Calc. % SiO_2 (z) in silicic acid. Calc. quantity of silicic acid (x) required for 5.8 g column from equation: $x = 3.384 \times 100/z$. Quantity of H_2O (y) required for column is $5.80 - x$.

(**b**) *Isooctane.*—Practical grade 2,2,4-trimethylpentane, 99.5+ %, bp 98–100°.

(**c**) *Isooctane-chloroform solvent mixture.*—Add 40 ml $CHCl_3$ to 1 L isooctane and mix. Store in air-tight bottle. (Do not use rubber stopper.) Soln contains ca 3.85% $CHCl_3$.

(**d**) *Coumarin std soln.*—1 mg/ml. Accurately weigh 100 mg coumarin into 100 ml vol. flask, dissolve in 50 ml $CHCl_3$, and dil. to vol. with isooctane.

(**e**) *Ethyl vanillin std soln.*—Prep. as in (d), using Et vanillin.

(**f**) *Vanillin std soln.*—Prep. as in (d), using vanillin.

19.017 ***Determination of Absorptivities***

Pipet 1 ml vanillin std soln into 100 ml vol. flask, add 3.4 ml $CHCl_3$, dil. to vol. with isooctane, and mix. Det. A at 270 and 325 nm against solv., (c), as ref. in the 1 cm silica cells. Calc. a (g/L: 1 cm) for vanillin at 270 and at 325 nm from equation: $a = 100A$.

Det. a for Et vanillin and vanillin similarly.

19.018 ***Preparation of Chromatographic Column***

Pack small cotton wad in bottom of *dry* chromatgc tube. To x g silicic acid in mortar add from buret y ml H_2O, mix thoroly and quickly to uniform powdery consistency with pestle, and immediately add 25 ml solv., (c). Mix and rapidly pour slurry thru funnel into tube. Rinse mortar and funnel with small vol. solv. Remove any air bubbles formed by stirring with long thin glass rod. Pack column with ca 2 lb/sq in. air pressure until bottom of meniscus of free solv. just touches top surface of silicic acid but outer part of meniscus is still clearly visible. *Immediately release pressure.* (*Important:* If column channels or cracks, discard. During packing and thereafter, keep column vertical. Tipping ruins column for further use altho it may appear normal.) Carefully add 15 ml solv. down side of tube with aid of glass rod so column is not disturbed. Drive solv. thru column. Washed column is now ready for calibration.

19.019 ***Calibration of Column***

Pipet and combine 1 ml of each std soln, (d), (e), and (f), in 25 ml vol. flask. Dil. to vol. with isooctane and mix. Pipet 2 ml soln down one side of chromatgc tube onto top of column. Drive soln into column with ca 2 lb air pressure and collect eluate in 10 ml graduated cylinder. Release pressure when bottom of meniscus touches top of column and outer part of meniscus is still clearly visible. Pipet 1 ml solv., (c), down same side of tube onto column and drive into column. Repeat with 2 addnl 1 ml portions solv. Fill tube to within 1″ of top with solv. Drive solv. thru column at rate of 5 ml/2–2.5 min, collecting 5 ml eluate fractions, alternating two 10 ml graduated cylinders during collection. Pour fractions into sep. test tubes in rack, numbering fractions consecutively. Drain cylinder before reusing by inverting on towel. Collect 10 fractions and det. A at 270 and 325 nm against solv., (c), as ref. in 1 peaks 1 and 2. Peak 7 is always highest peak in pure vanilla samples. Sum of 8 peak hts, calcd on basis of internal std peak ht = 1.00, provides useful information on quantity of vanilla acids present. Ratio peak 2:peak 7 also provides significant information. Note also presence or absence of foreign peaks. If some peaks are too high for measurement, repeat detn with greater attenuation.

(*Note*: TMS derivatives tend to break down with heat, and continued use may impair column efficiency. Also, artifacts occur sometimes, particularly on first run of day. Columns usually can be reconditioned by overnight heating at 250° with slow stream of He. Disconnect column from detector when performing such reconditioning. Special regenerating liqs (e.g., Silyl-8, Pierce Chemical Co.) are also available for injection into columns used for TMS work. Heating thermal conductivity detectors at higher temps, without current, helps keep them free from decomposition products.)

LEMON, ORANGE, AND LIME EXTRACTS AND FLAVORS

19.049 Specific Gravity—Official Final Action

Det. sp gr at 20/20° with pycnometer as in 9.011.

19.050 Alcohol (*17*)—Official Final Action

(Applicable to exts consisting only of oil, alcohol, and water)

Det. sp gr at 15.56/15.56° or at 20/20° as in 9.011 and oil content as in 19.060, 19.061, or 19.116, and apply following formula: Let S represent sp gr of sample; O, sp gr of oil; and p, % oil found. Then $100 - p$ = % H_2O-alcohol soln, sp gr of which, represented by P, is calcd as follows:

$$S = [Op + P(100 - p)]/100;$$

therefore

$$P = (100S - Op)/(100 - p).$$

Det. E, alcohol equiv. of P, from 47.003. It gives % alcohol in alcohol-H_2O soln. To find % alcohol in ext, apply following formula:

$$\% \text{ by vol. of alcohol in ext} = E(1 - p/100).$$

Value of O for lemon oil may be taken as 0.86 and for orange oil as 0.85.

19.051 ★ Methanol—Official Final Action ★

See 19.047, 10th ed.

Isopropanol—Official Final Action

Applicable to Lemon Extract in Absence of Acetone (18)

19.052 ***Preparation of Sample***

Place sample contg ≤8 g total alcohols (approximation of alc. content may be made from sp gr detn and ref. to 47.003), into separator contg in stem cotton pledget wet with H_2O. Add 25 ml 10% NaCl soln and 25 ml pet ether. Shake well and when layers sep. drain lower layer into flask. Repeat extn with 3 addnl 25 ml portions NaCl soln or until alcohol is completely extd. Add H_2O to combined aq. exts until vol. is ca 150 ml. Connect flask to vertical condenser and distill into 100 ml vol. flask, removing flask when distillate is 2–3 ml below mark. Dil. to vol. and mix.

19.053 ***Qualitative Test for Acetone***

To 2 ml distillate add 0.5 ml *5% alc. o-nitrobenzaldehyde soln* and 1 ml 10% NaOH soln. Mix; then shake with small quantity of $CHCl_3$. If $CHCl_3$ turns blue, acetone is present.

19.054 ***Determination***

Pipet 10 ml distillate into 500 ml erlenmeyer contg 50 ml ca 2N $K_2Cr_2O_7$ and add 100 ml H_2SO_4 (1 + 3). Stopper flask, swirl, and let stand 30 min. Add 100 ml 30% $FeSO_4 \cdot 7H_2O$ soln. Connect flask to vertical condenser thru foam trap. Slowly distill ca 100 ml into 500 ml vol. flask contg 200–300 ml cold H_2O. Dil. to vol., mix, and pipet 25 ml into g-s flask contg 25 ml 1N NaOH; add 50 ml stdzd 0.1N I with swirling. Let stand 15 min. Add 26 ml 1N HCl and at once titr. residual I with stdzd 0.1N $Na_2S_2O_3$, adding starch soln when I color is nearly discharged. Each ml 0.1N I consumed in reaction = 1.001 mg isopropanol.

Applicable to Lemon and Orange Flavors in Presence of Acetone (19)

19.055 ***Apparatus***

Glassware.—Use foil wrapped stoppers or preferably all-glass still. Provide condenser with adapter which reaches several inches into vol. flask.

19.056 ***Preparation of Sample***

Proceed as in 19.052, placing 100 ml vol. flask in ice-H_2O bath.

19.057 ***Determination of Acetone***

Pipet aliquot preferably contg 0.1–0.3 g acetone into 100 ml vol. flask and dil to vol. with H_2O. Det. A at 265 nm with H_2O as ref. soln in Beckman Instruments Model DU spectrophtr or equiv. instrument. Correct for A of H_2O in same cell as used for sample, if necessary. Det. quantity of acetone in the 100 ml vol. flask by ref. to std curve prepd from redistd acetone.

In absence of purified acetone, g acetone/100 ml may be estd from equation: $C = A/3.08$, where C = g acetone/100 ml, A = corrected A in 1 cm cell, and 3.08 = assumed A of 1 g/100 ml soln of acetone in 1 cm cell. Calc. to g acetone/100 ml sample.

19.058 ***Determination of Isopropanol***

Proceed as in 19.054, distg ca 100 ml into 250 ml vol. flask contg ca 100 ml cold H_2O and held in ice-H_2O bath. Dil. to vol. with H_2O and det. corrected most of aq. layer seps. (Small layer of emulsion may remain between layers.)

Drain aq. layer into flask, retaining any emulsion in separator. Keep flask and separator stoppered between addns to avoid contact with air. Add ca 100 ml H_2O to separator, shake vigorously, and drain entire contents into 250 ml centrf. bottle. Stopper, and centrf. until 2 well-sepd layers are obtained. Blow off lower layer in centrf. bottle, using expeller, into flask contg aq. fractions previously sepd, add ca 0.2 ml phthln and titr., using std 0.2*N* HCl. As end point approaches, repeat addn of indicator and titr. to disappearance of pink. (Liq. becomes white or grayish.)

Conduct blank detn similarly, using same amts of all reagents. Subtract titrn of sample from that of blank to obtain equiv. of 0.2*N* alkali consumed. 1 ml 0.2*N* alkali = 39.2 mg esters as linalyl acetate.

Pinene (31)—Official Final Action

19.090 *Qualitative Test*

(*Caution:* Ethyl nitrite may be harmful. Avoid contact with skin and breathing vapor.)

Mix 10% distillate, 19.077, with 5 ml HOAc, cool mixt. thoroly in freezing bath, and add 10 ml *Et nitrite.* Add 2 ml HCl (2 + 1) slowly with constant stirring. Keep mixt in freezing bath 15 min. Collect crystals formed on filter, using suction, and wash with alcohol. Return combined filtrate and washings to freezing bath 15 min. Collect addnl crystals formed on original filter. Wash combined crops of crystals thoroly with alcohol. Dry at room temp. and dissolve in min. quantity of $CHCl_3$. Add MeOH to $CHCl_3$ soln, little at time, until nitrosochlorides crystallize out. Mount sepd and dried crystals in olive oil and examine under microscope. Pinene nitrosochloride crystals have irregular pyramidal ends; limonene nitrosochloride crystallizes in needles.

ALMOND EXTRACT

Alcohol—Official First Action

19.091 *Method I (32)*

Fill 50 ml pycnometer with sample at 15.56°, and empty into separator contg ca 10 g NaCl. Wash out pycnometer several times with satd NaCl, using total of ca 100 ml. Ext twice with 50 ml portions pet ether (bp 40–60°). Collect pet ether ext in second separator and wash with two 25 ml portions satd NaCl soln. Combine original NaCl soln with washings, add little *powd pumice*, and distill into 100 ml pycnometer (Fig. 9:1). When almost 100 ml collects, dil. to vol. with H_2O at convenient temp. and det. alcohol from sp gr as in **9.013**, using table, **47.003.**

19.092 *Method II (33)*

Det. sp gr of ext at 15.56/15.56° or at 20/20° as in 9.011 and benzaldehyde content as in 19.093. Apply formula given in 19.050, using benzaldehyde content as % oil found.

Benzaldehyde

19.093 *Gravimetric Method (34)—Official First Action*

Measure 10 ml sample into each of two 300 ml erlenmeyers and add 10 ml *phenylhydrazine soln* (3 ml HOAc, 40 ml H_2O, 2 ml phenylhydrazine) to one flask and 15 ml to other. Let mixts stand overnight in dark place.

Add 200 ml H_2O and filter thru weighed gooch provided with thin layer of asbestos. Wash ppt first with cold H_2O and finally with 10 ml 10% alcohol. Dry 3 hr at 70° at pressure $\leq$100 mm Hg or to constant wt over H_2SO_4. Wt ppt $\times$ 5.408 = wt benzaldehyde in 100 ml sample. If the 2 detns do not agree, repeat operation, using larger quantity phenylhydrazine soln.

Alternative Gravimetric Method (35)—Official Final Action

19.094 *Reagent*

2,4-Dinitrophenylhydrazine soln.—Add 50 ml alcohol to 3.0 g 2,4-dinitrophenylhydrazine. Slowly add 10.0 ml H_2SO_4 while stirring. After reagent dissolves, add addnl 40 ml alcohol and filter thru Whatman No. 12 paper.

19.095 *Determination*

Measure sample contg ca 10–50 mg benzaldehyde (ca 5 ml flavors, 100–200 ml cordials) into distn flask. Add enough alcohol to ensure $\geq$10% by vol. in distillate and dil. to ca 150 ml for flavors and 250 ml for cordials with H_2O. Distill ca 100 ml flavors and 200 ml cordials and collect in vol. flask in ice bath. Transfer distillate to 600 ml beaker (also in ice bath) with 100 ml chilled alcohol. Add 25 ml H_2SO_4, mix thoroly, and immediately add 25 ml 2,4-dinitrophenylhydrazine soln, while stirring. Heat on steam bath or hot plate 30 min at ca 75°, stirring occasionally (avoid boiling).

Remove from heat, let ppt settle, and filter by decanting most of supernatant thru weighed gooch prepd with thin asbestos mat before transferring bulk of ppt. Wash ppt with ca 25 ml H_2O at room temp. or below.

Dry at 100° to constant wt (ca 2 hr). Wt ppt $\times$ 0.3707 = wt benzaldehyde.

Ultraviolet Spectrophotometric Method (35)—Official Final Action

19.096 *Reagents and Apparatus*

(a) *Spectrophotometer.*—Quartz spectrophtr, Beckman Instruments Model DU, or equiv., with UV sensitive phototube and H lamp.

(b) *Benzaldehyde.*—Redistd; sp gr 1.041–1.046.

(c) *Alcohol.*—Reagent grade alcohol or MeOH.

(d) *Benzaldehyde std soln.*—Weigh 1 g benzaldehyde into 100 ml vol. flask and dil. with alcohol. Transfer 1 ml of this soln to 100 ml vol. flask, using 10% alcohol. Dil. 1, 2, 4, 6, 8, 10 ml aliquots to 100

Fd Cosmet. Toxicol. Vol. 3, pp. 311–324. Pergamon Press 1965. Printed in Great Britain

Review Section

Chronic Toxicity of Essential Oils and Certain Other Products of Natural Origin*

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ESSENTIAL OILS

Definition of essential oils (ethereal oils, aetherolea)

The essential oils are a group of odorous principles which are soluble in ethanol but only to a limited extent in water. Chemically they are mixtures of esters, aldehydes, alcohols, ketones, and terpenes. If exposed to the air, oxidation occurs and it is normal practice to add antioxidants, usually either (1) propyl, octyl or dodecyl gallates, or (2) butylated hydroxyanisole, to them at a concentration of 0·1%.

Chemistry of essential oils

The major and more important minor constituents of the essential oils have been known for many years, but following the introduction of vapour phase chromatography the list of substances known to be present in small or trace amounts has increased and is still increasing. The same constituent, such as α-pinene, may be present in a great variety of oils either as a major or minor constituent. Similarly, there may be distinct differences between oils derived from different species within the same genus (e.g. Eucalyptus).

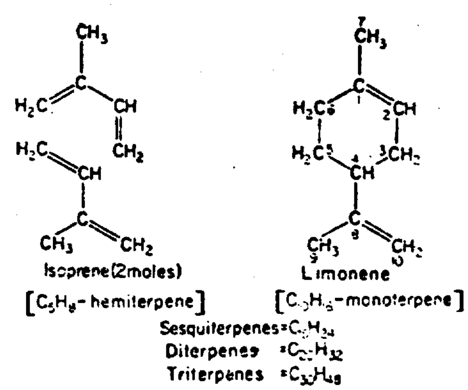

FIG. 1. Isoprene as a building block of terpenes.

Terpenes and terpene derivatives—alcohols, aldehydes, ketones and esters—are the chief constituents of all essential oils. It is generally agreed that isoprene is the 5-carbon molecule from which the several types of the terpene molecule are built (Fig. 1). Under warm

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conditions in the presence of oxygen, oxidation may occur in the laboratory. In the natural state either oxidation or reduction may proceed. Thus the variety of alcohols, aldehydes, ketones and esters emerge (Figs. 2, 3 and 4). The terpene hydrocarbons are not

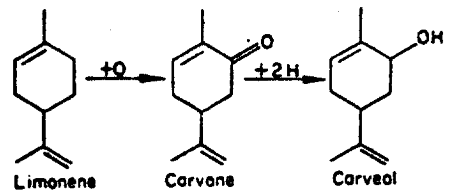


FIG. 2. Oxido-reduction from limonene to carvone and carveol.

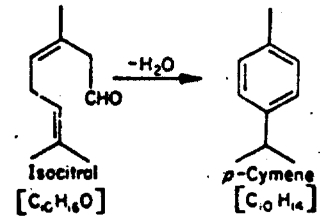


FIG. 3. Oxidation of isocitral in lemon oil to p-cymene.

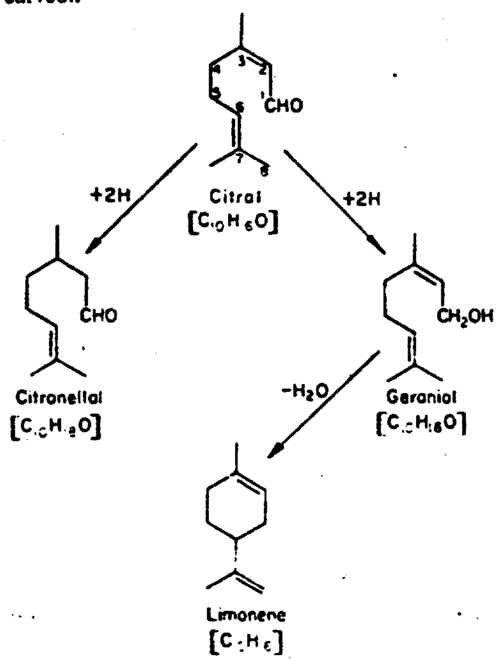


FIG. 4. Relationship of various constituents of essential oils to each other.

devoid of odour, but in most oils it is the other constituents, usually minor constituents, which are responsible for their characteristic scents.

Pharmacology and uses of essential oils

Taken internally it is a general property of essential oils that they are mildly irritant to the mucous membrane of the mouth and digestive tract. Their ingestion gives rise to a sensation of warmth and increased salivation. Because of this property they are used as aids to appetite and digestion. Following ingestion the main constituents are secreted via the lungs, kidneys and skin. In the lungs they are slightly antiseptic and act to stimulate respiration and cardiac activity. Their administration gives rise to a transient rise in blood pressure, and because of this they have been used in conjunction with more powerful stimulants in the treatment of syncope. Taken after meals they have a carminative action, and certain essential oils (e.g. oil of dill) are used to counteract the griping pains of colic.

When large amounts of some of the essential oils are ingested and there is increased excretion of constituents via the kidneys, irritation of the kidneys, bladder and urethra may occur. It is recognized that, where pre-existing inflammatory conditions of the urinary tract are present, they may be aggravated by small doses of ingested oils.

An irritant and rubefacient effect is observed following the application of many of the essential oils to the skin. Typically there is a smarting sensation followed by mild anaesthesia. They have therefore been used medicinally as counter-irritants, although a danger of blistering is recognized.

Inhalation of the oils is followed by the arrest of profuse secretion within the respiratory tract. Thus they have been used to relieve bronchiolar congestion in chronic bronchitis.

It is as pleasing odorous principles that the essential oils are best known to us. We encounter them as flavouring agents and as scents in soaps and cosmetics of all kinds, and in insect repellents. For these purposes they are present in low concentrations and are for the main part applied externally only.

Finally, large quantities of essential oils and products derived from them are used by industry, especially as constituents of paints and varnishes, as disinfectants, in mineral flotation and as solvents. There is a growing tendency for synthetic chemicals to be used in the place of crude or more or less refined essential oils as used in the past. However, it is the essential oils themselves which usually provide the starting materials for the syntheses involved.

Chronic toxicity of essential oils

As we have seen, irritation of the skin, respiratory and urinary tracts may all be attributable to excessive exposure to essential oils.

The questions which faced us in 1959 were: could these irritant effects under certain circumstances lead to cancer induction, or could the oils act as co-carcinogens or tumour-promoting agents?

The substances studied by us were the following:

Oils. Orange, lemon, lime, grapefruit, bergamot, eucalyptus, peppermint, clove, cinnamon, cedarwood, turpentine.

Constituents. α-Pinene, phellandrene, *l*-decene, linalool, terpeneless fraction of orange oil, terpene fraction of orange oil, *d*-limonene, *n*-decyl aldehyde, *d*-carvone, eugenol, terpineol, linalyl acetate, terpinyl acetate, citral.

Preliminary tests

As a preliminary to the main experiments we examined the early effects of these substances on mouse skin. Mice of the '101' inbred strain and approximately 8–10 weeks of age were used for this purpose. The dorsal hair was removed by electric clippers and the substances applied either in undiluted form or at various concentrations in acetone. Two applications of the test material were made 7 days apart. A specimen of dorsal skin was obtained by biopsy 3 days after each application.

In these preliminary tests most of the substances mentioned above gave rise to moderate or marked epidermal hyperplasia. In some cases areas of necrosis with ulceration, weeping and crusting were seen. High concentrations of the oils in acetone were often more irritant to mouse skin than the undiluted oils.

In general, the terpene hydrocarbons proved to be irritant to mouse skin, whereas the alcohols, aldehydes, ketones and esters were systemically toxic and could only be applied as dilutions in acetone. Similarly, oils wherein the main constituents are alcohols or esters (e.g. clove oil, which contains more than 70% eugenol) were particularly toxic to mice when applied to the skin. An exception to this generalization was seen in the case of peppermint oil, which contains approximately 60% of menthol (Fig. 5) and was neither systemically toxic nor locally irritant. Similarly, cedar-wood oil, the chief components of which are cedrene (80%) (Fig. 5) and cedrol (3–14%), was also without either systemic or local effect.

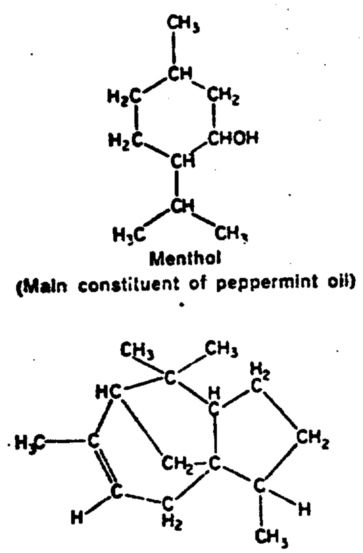


FIG. 5. Examples of essential oils lacking systemic or local effects.

Skin tumour-promotion by citrus oils

A full report of the experiments in which we observed the promotion of benign and malignant skin tumours by the repeated application of different citrus oils has been presented elsewhere (Roe & Peirce, 1960). Here we give a brief resumé of our findings.

Mice of two different strains were used, '101' strain (inbred) and stock albino (random-bred) and experiments were started when mice were approximately 8 weeks of age. In the case of the test groups, treatment began with a single application of 3,4-benzopyrene (BP), 9,10-dimethyl-1,2-benzanthracene (DMBA), or urethane to the whole of the dorsal skin after removal of the hair by electric clippers. These substances were applied to the skin in acetone solution, the dose being sufficient to initiate skin tumour formation but, generally speaking, inadequate for complete carcinogenesis (Berenblum & Shubik, 1947 a,b 1949; Salaman & Roe, 1953). No further treatment was given for a period of 3 weeks, after which the test substance was applied once weekly, either in undiluted form or diluted with acetone. Control groups received either the initial treatment alone, or treatment with the test substance following an initial application of acetone only. Dorsal hair was removed repeatedly as necessary throughout the experiment. Benign warts (papillomata) and malignant skin tumours (epitheliomata) appeared in some of the groups which received both pretreatment with a subcarcinogenic dose of BP, DMBA, or urethane and repeated treatments with one of the test substances. Occasional tumours only, all of them benign, were seen in the control groups. The results are depicted in Tables 1, 2 and 3.

Table 1. *Tests for tumour-promotion by citrus oils*

Primary treatment with DMBA* (given as single application in 0·2 ml acetone) (μg)	Secondary treatment (0·25 ml once weekly, starting 3 weeks after primary treatment)	Skin tumour incidence (33 weeks after start of secondary treatment)	
		No. of surviving mice with papillomas	Total no. of papillomas
300	Orange oil (either undiluted or at 80% or 40% concentration in acetone)	28/43	83
None	Do.	1/48	1†
300	None	5/38	6†
225	Lime oil (undiluted)	8/14	47
None	40% Lime oil in acetone	0/15	0
225	Bergamot oil (undiluted)	0/10	0
None	Do.	0/8	0
300	Lemon oil (undiluted)	10/15	38
300	Grapefruit oil (undiluted)	13/15	37

*9,10-Dimethyl-1,2-benzanthracene.

†Tumours on skin, outside treated area.

Table 2. *Tests for tumour-promotion by the two main fractions of orange oil*

Primary treatment with DMBA (given as single application in 0·2 ml acetone) (μg)	Secondary treatment (0·25 ml once weekly, starting 3 weeks after primary treatment)	Skin tumour incidence (33 weeks after start of secondary treatment)	
		No. of surviving mice with papillomas	Total no. of papillomas
300	None	1/16	1
300	80% Terpene fraction* of orange oil	8/15	29
300	20% Non-terpene fraction* of orange oil	1/13	1
None	Do.	0/34†	0

*Approximately 95% of orange oil finds its way into the terpene fraction and 5% into the non-terpene fraction. Hence, if the tumour-promoting activity of the oil were due to the activity of the non-terpene fraction a strong positive result should have been obtained in the third group.

†One animal had a melanotic tumour (naevus-type) in subcutaneous tissues of treated area; and another had a small haemangioma in the same region.

Table 3. *Tumour-promotion by eucalyptus and turpentine oils and certain constituents of essential oils*

Group*	Primary treatment with DMBA (given as single application in 0·2 ml acetone) (μg)	Secondary treatment (0·25 ml once weekly, starting 3 weeks after primary treatment)	Skin tumour incidence (33 weeks after start of secondary treatment	
			No of surviving mice with papillomas	Total no. of papillomas
1	300	Undiluted oil of turpentine	8/19†	10†
2	300	None	1/16‡	1‡
3	225	Undiluted oil of eucalyptus	4/14	5
4	225	None	0/13	0
5	150	40% α-Pinene	3/15	4
6	150	40% Phellandrene	2/17	2
7	150	40% 1-Decene	7/17	13

*Groups 1 and 2 were contemporaneous, so also were groups 3 and 4. No control group treated with 150 μg DMBA only was set up in parallel with groups 5, 6 and 7. However, it is extremely unlikely that tumours would have arisen in response to this treatment only.
†One mouse had a single papilloma outside the treated area.
‡Single papilloma outside the treated area.

Tumours at the urethral orifice of female mice treated with orange oil

In our earlier report (Roe & Peirce, 1960) we described the induction and appearance of tumours at the urethral orifice of female mice during treatment with orange oil with or without DMBA pretreatment, or with the terpeneless fraction of orange oil (Table 4).

Table 4. *Incidence of tumours of the urethral orifice of female* '101' *strain mice treated with orange oil*

Treatment	Incidence of tumours of the urethral orifice in female survivors
FIRST SET OF EXPERIMENTS	
DMBA + Orange oil to dorsal skin	4/40
Orange oil only to dorsal skin	2/40
DMBA only to dorsal skin	0/38
DMBA + terpene fraction of orange oil to dorsal skin	0/10
DMBA + terpeneless fraction of orange oil to dorsal skin	1/10
Total......	7/138
SECOND SET OF EXPERIMENTS	
Terpeneless fraction of orange oil only to dorsal skin	0/34
Terpeneless fraction of orange oil applied directly to urethral orifice	0/16
225 μg DMBA applied to dorsal skin, then terpeneless fraction of orange oil (0·05 ml by stomach tube) once weekly	0/20

Altogether we saw 7 of these tumours, all in mice of the '101' strain and all in mice treated with a particular batch of orange oil. No such tumours arose in subsequent experiments where other batches of orange oil were used, nor in experiments with other citrus oils or citrus oil constituents. Deliberate painting of the urethral orifice of female '101' strain mice with the terpeneless fraction of orange oil did not elicit any tumours (Table 4: second set of experiments). Similarly, no urethral tumours arose in female mice painted with the terpeneless fraction repeatedly without DMBA pretreatment, nor in mice given a single application of DMBA to the skin followed by the terpeneless fraction repeatedly by stomach tube.

The tumours were benign squamous papillomas, often mitotically active but never invasive. Some grew to a size of almost 1 cm in diameter. All were heavily infiltrated by polymorphonuclear leucocytes. Figs. 6 and 7 illustrate the macroscopic and microscopic appearances of these tumours, respectively.

Looking back it would seem likely that a second factor was involved in the genesis of the urethral tumours; perhaps an intercurrent genito-urinary infection in the mice used in the earlier experiments; perhaps a slight dietary difference. Alternatively, one must suppose that the earlier and later batches of orange oil differed in some material way. It is perhaps relevant that '101' strain mice are especially susceptible to papillonephritis, a disease of unknown aetiology which initially causes necrosis and calcification of the renal papilla (there is only one papilla in the mouse kidney) and subsequently retrograde changes in the renal cortex leading eventually to renal failure. However, this disease was seen in both the earlier and later experiments with orange oil. A second possibly relevant fact is that in the earlier, but not the later, experiments referred to above, intussusception and anal prolapse were common causes of intercurrent deaths. We have no idea why this should have been. The fact is that, though we wished to study the mechanism of induction of these urethral tumours further, we could not do so because we had altogether lost the knack of inducing them.

Experiments on other essential oils

Weak tumour promotion was observed with turpentine oil and with α-pinene, one of its principle constituents (Table 3, Fig. 8). Previously, Mackenzie & Rous (1941) reported that

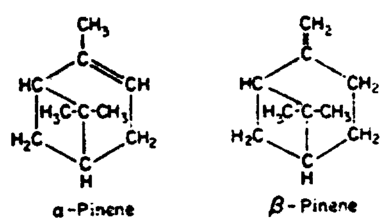


FIG. 8. Main constituents of turpentine oil (average for several varieties of oil). α-Pinene 62%; β-pinene 33%; alcohols, aldehydes, esters, etc. 5%.

turpentine oil promoted skin tumour development in rabbit skin, but Berenblum (1941) and Shubik (1950) found it to be more or less ineffective in the mouse. It is likely that the composition of the oils used in the various experiments was different, and this may explain the disagreement between our findings and those of Berenblum (1941) and Shubik (1950).

The eucalyptus oil tested by us appeared to have weak promoting activity for mouse skin (Table 3). Four out of 14 mice which survived 33 weeks or more developed skin tumours in response to a single application of 225 μg DMBA followed by once-weekly applications of undiluted eucalyptus oil. Malignant skin tumours were seen in 3 of these 4 mice, the first after only 14 weeks of secondary treatment. Mice treated alone with 225 μg DMBA developed no skin tumours during a comparable period of observation. Phellandrene, one of the major constituents of eucalyptus oil, applied at a concentration of 40% in acetone, also had a weak promoting effect.

Bergamot oil was less irritant than the other citrus oils in the preliminary skin tests and proved inactive as a tumour-promoting agent. In fact, this result fits in with the general thesis that the terpene hydrocarbons are responsible for both the irritant and tumour-promoting effects. Whereas the other citrus oils mentioned above, orange, lemon, grapefruit, etc. contain approximately 90% terpene, 60–70% of bergamot oil consists of alcohols and esters. On the other hand, in another test, linalool as a 20% solution in acetone elicited a weak tumour-promoting response. Linalool is one of the principle alcohols in bergamot.

Tumour-promotion and tumour-induction by citrus oils in the forestomach epithelium of mice

A preliminary report has been made of results of the first tests of the activity of lime oil on the forestomach epithelium of mice (Peirce, 1961) and a full report of all the experiments has been submitted for publication elsewhere (Field (née Peirce) & Roe, 1965). Here we will communicate only a brief summary of the findings.

The object of our experiments was twofold. Firstly to see whether two-stage carcinogenesis could be demonstrated in tissues other than the skin and secondly, to see whether citrus oils act as tumour promoters in the gastro-intestinal tract.

Previous attempts to induce tumours of the stomach in mice by "small" doses of polycyclic hydrocarbons followed by croton oil (Berenblum & Haran, 1955) failed, possibly because the dose of the hydrocarbon was excessive in view of the high susceptibility of the epithelium of the mouse forestomach to tumour-induction (Bock & King, 1959). In her experiments, therefore, Peirce (1961) tested various much lower doses of BP or DMBA, either alone or followed by repeated treatments with lime oil. The technique was as follows: mice, when 6–8 weeks old, were starved overnight and given the requisite dose of BP or DMBA dissolved in 0·05 ml of polyethylene glycol (PEG of average mol wt 400). Three weeks later secondary treatments were begun. These consisted of once-weekly doses of 0·05 ml undiluted lime oil, or no treatment, following overnight starvation. The only tumours observed in the gastro-intestinal tract were in the stomach. Tumours of the

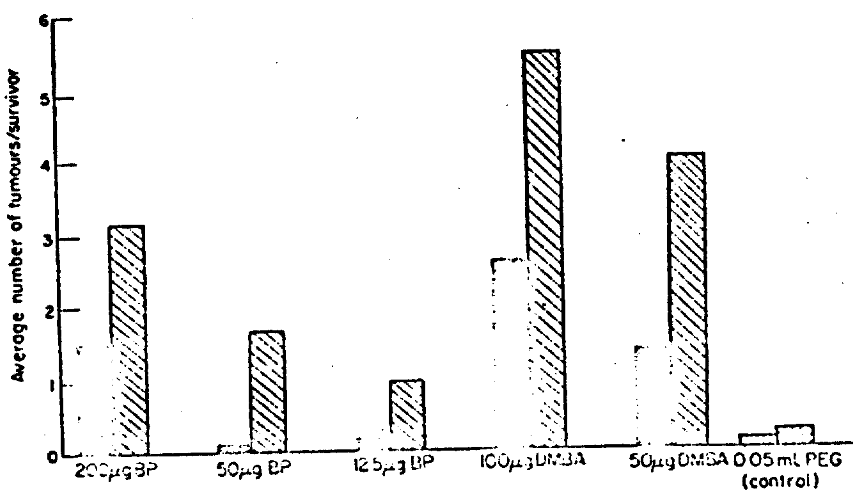


FIG. 9. Tumour-promoting effect of 40 once-weekly treatments with lime oil after various initiating doses of 3,4-benzopyrene (BP) or 9,10-dimethyl-1,2-benzanthracene (DMBA) in the mouse forestomach. ▭, initiator alone; ▭, initiator+promoter; ▭, polyethylene glycol solvent control; ▭, control+promoter.

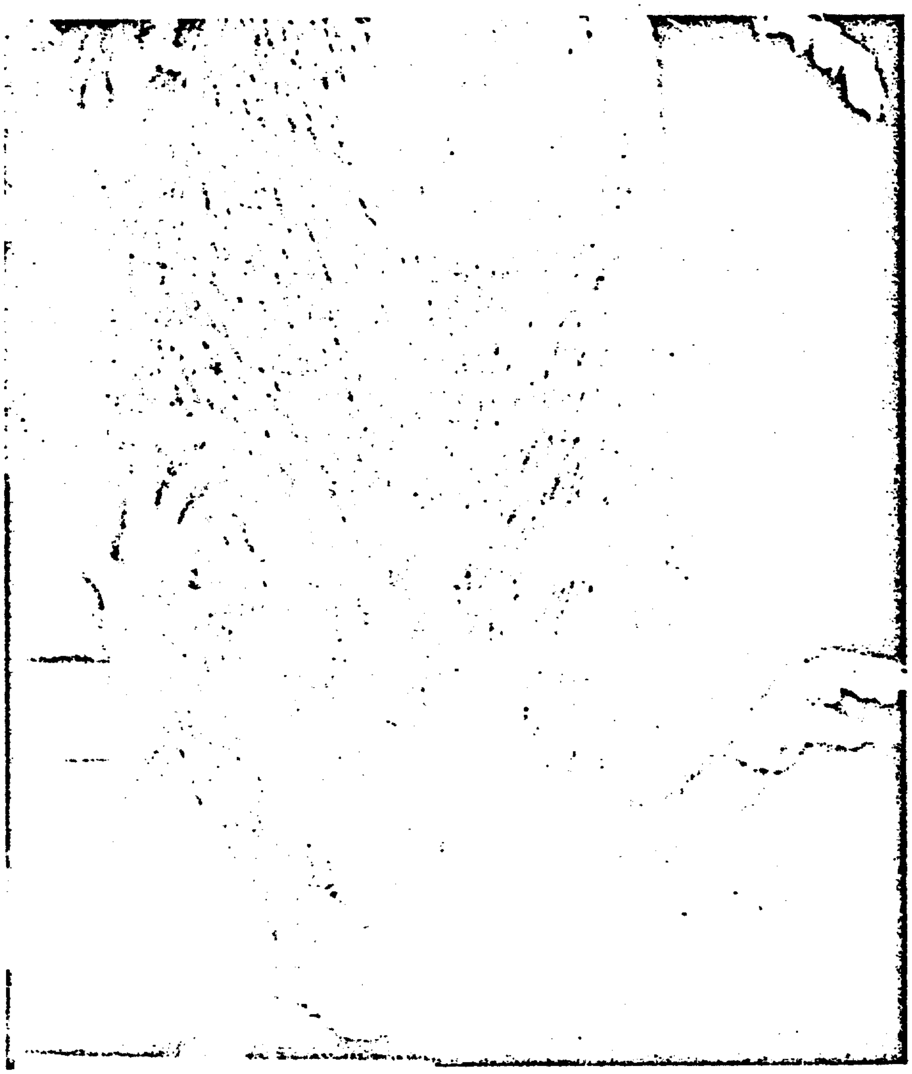


Fig. 6. Papillomatous tumor of urethral orifice in a female mouse of strain 101 treated with a single application of 300 μg. DMBA followed by 15 once weekly applications of 40% orange oil in acetone. (Reprinted by kind permission of the Editors of the *Journal of the National Cancer Institute*).

FIG. 7. High-power view of same section. *Note* inflammatory infiltration by polymorphonuclear cells. ×85. (Reprinted by kind permission of the Editors of the *Journal of the National Cancer Institute*).

glandular stomach were infrequent and their occurrence showed no relation to treatment. Forestomach tumours arose in response to treatment with BP alone or DMBA alone, particularly at the higher dose levels. However, secondary treatment with lime oil for periods up to 40 weeks consistently and significantly increased the incidence of forestomach tumours (Fig. 9).

In mice killed at the cessation of 40 weeks of secondary treatment the vast majority of tumours were benign papillomas. However, malignant tumours were seen occasionally before this time and more frequently in animals allowed to live longer. Every one of 12 malignant forestomach tumours, all examined histologically and all showing penetration of the muscular coats of the stomach, arose in animals treated with both hydrocarbon and lime oil: none were seen in comparable mice treated with hydrocarbon only. Transperitoneal spread and distant metastases were observed in some cases.

Occasional benign, but no malignant, tumours were seen in mice treated with PEG and no further treatment, or PEG followed by lime oil (Fig. 9).

Urethane dissolved in water followed by lime oil once weekly gave rise to a few forestomach papillomas, whereas urethane without subsequent treatment was almost ineffective.

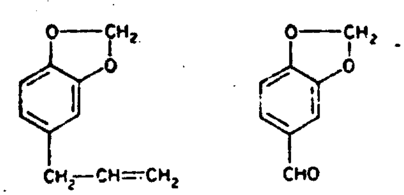
Heating the lime oil under a reflux condenser for 3 hr did not abolish its tumour-promoting activity for the forestomach epithelium.

In other experiments lime oil was incorporated in the diet of mice and comparisons were made between animals receiving 50 μg BP by stomach tube and then fed diets containing various levels of lime oil or no lime oil at all. The presence of lime oil in the diet at all levels (0·5, 2 or 8 ml/kg) increased the incidence of papillomas of the forestomach. In this experiment there was no effect on the incidence of malignant forestomach tumours.

A less clear-cut result was obtained in mice given "orange squash" instead of drinking water following a single dose of 50 μg BP. In this case treatment with squash appeared to increase the incidence of forestomach tumours, but the extent of the increase bore no relation to the concentration of orange oil in the squash. A subsequent experiment with orange squash, using a different strain of mice, gave a negative result.

Carcinogenicity of safrole

Another component of many essential oils but especially of sassafras oil, namely safrole (Fig. 10) has recently come into the news as a possible environmental carcinogen. Safrole is a major constituent of sassafras oil, star anise oil and camphor oil, and a minor con-



Safrole

Heliotropin (Piperonal)

FIG. 10. Possible carcinogenic components of essential oils.

stituent of oil of nutmeg and cinnamon leaf oil. It has been used widely as a flavouring agent in 'root beer' (a beverage consumed in large volumes in North America), in chewing gum, toothpastes and certain pharmaceutical preparations. Safrole itself, but more frequently heliotropin, a related aldehyde, is used to scent soaps and cosmetics.

Homburger, Kelley & Friedler (1961) reported the induction of hepatic tumours in rats fed safrole at 1% in the diet. Tumour-induction was preceded by cirrhosis and the accumulation of massive deposits of ceroid pigment. Most of the tumours were benign. Occasionally adenomata were seen in the absence of cirrhosis. Tumours were more prominent in animals fed a casein-supplemented than a protein-deficient diet. Long, Nelson, Fitzhugh & Hansen (1963) were able to confirm the findings of Homburger *et al.* (1961) using only a 0·5% dietary level of safrole.

NATURAL PRODUCTS OTHER THAN ESSENTIAL OILS

An investigation of certain euphorbia latices

When we first began our investigation of the biological activity of the euphorbia latices we did not realize that there was a possible relationship between them and the essential oils (*vide infra*).

Dr. J. Fawcett at the London Hospital drew our attention to the fact that some of the euphorbia latices are highly irritant for the human skin and mucous membranes. He thought it might be of interest to test them for tumour-promoting activity since *Croton tiglium*, which provides croton oil, is of the euphorbia family.

Most members of the euphorbia family have it in common that when their stems are cut a thick white latex exudes and eventually coagulates. During the war, when Britain was deprived of its source of natural rubber by the Japanese occupation of Malaya, the latex of *E. ingens* was examined to see if it could act as a substitute. However, it proved too irritant to handle on a large scale.

In 1959, we tested the latex of *E. ingens* on mouse skin (Roe & Peirce, 1961; Peirce & Roe, 1962). It evoked marked hyperplasia and ulceration of the epidermis at concentrations above 1% in acetone. At 1% and lower concentrations it caused hyperplasia without necrosis. A 1% solution in acetone proved to be highly effective as a tumour-promoting agent (Table 5).

Table 5. *Tumour-promotion by latex of* Euphorbia ingens *in* '101' *strain mice*

Initiating treatment (μg DMBA)	Promoting treatment	No. of surviving mice with papillomas	Total no. of papillomas
300	None	0/19	0
300	0·1–2% extract of latex of *Euphorbia ingens*	12/20	54
None	Do.	1/17	1

Subsequently, with the help of the Royal Botanic Gardens, Kew, London, we tested several other euphorbia latices both for irritant and tumour promoting effects on mouse skin. Several were found to be highly or moderately active in both respects. On the whole, the two types of activity went hand in hand. The most effective latex was that of *E. tirucalli* (Table 6).

Table 6. *Tumour promotion by various euphorbia latices for skin of mice*

Promoting treatment (1% acetone extract once weekly)	Hyperplastic changes in epidermis	No. of surviving mice with papillomas	Total no. of papillomas
	DMBA INITIATING TREATMENT (150 μg)		
None	—	0/20	0
E. tirucalli	++++	15/15	358
E. grandidens	+++	13/16	163
E. canariensis	+++	10/17	103
E. wulfenii	++	11/18	69
E. candelabrum	++	15/19	68
E. obovalifolia	++++	7/20	40
E. abyssinica	+	10/19	28
E. cooperi	+	3/18	4
E. triangularis	+	1/20	1
	NO INITIATING TREATMENT		
E. tirucalli	++++	2/20	2
E. grandidens	+++	0/17	0

The complete chemistry of these latices is not known but it is clear that we are once again involved with terpene chemistry. According to Warren & Watling (1958), two of the major constituents of the resin derived from *E.* tirucalli are triterpene alcohols (Fig. 11).

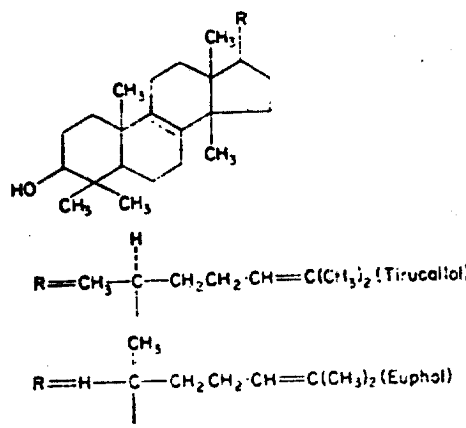


FIG. 11. Major constituents of resin of *Euphorbia tirucalli.*

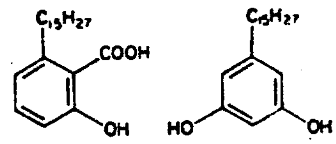
The discovery of the strong promoting activity of the euphorbia latices is especially interesting because it is the first time that agents of the same order of potency as croton oil have come to light.

Carcinogenicity of tannins

The late Professor Korpassy was the first to draw attention to the fact that hydrolysable tannins may give rise to cirrhosis and liver tumours in rats (Korpassy, 1961). Moreover Kirby (1960), at the Chester Beatty Institute, London, induced both liver tumours and tumours at the site of injection of three different condensed tannins. With three hydrolysable tannins, myrobalans, chestnut and valonea, liver tumours but no local neoplasms were obtained.

Tumour-promotion by cashew-nut shell liquid

The liquid obtained from the shells of cashew nuts contains phenolic substances of value as raw materials in the manufacture of resins, lubricants and plastics. Its main constituents are anacardic acid (90%) and cardol (10%) (Fig. 12).



Anacardic acid   Cardol

FIG. 12. Main constituents of cashew-nut oil.

The liquid is a cause of occupational dermatitis (Schwartz, Birmingham, Campbell & Mason, 1945), the irritant properties being associated mainly with cardol, which is chemically related to urushiol, the irritant factor in poison ivy.

We tested it in mice to see whether it caused epithelial hyperplasia. On finding that it did so we proceeded to test it for tumour-promoting activity.

Twenty '101' strain mice were given a single application of 150 μg DMBA and then once weekly applications of 3–5% cashew nut oil in acetone. Of 15 mice which survived for 20 or more weeks, 12 developed papillomas. A total of 41 papillomas arose in these 12 mice. We saw no malignant tumours in this particular experiment.

GENERAL DISCUSSION

Taking the essential oils together as a group it is clear that we have relatively little precise knowledge regarding the toxicity of many of their major constituents and next to none regarding the majority of their minor constituents or oxidation products.

The fact that most of the oils have been used through the centuries by man as flavourings, aids to digestion, perfumes and medicines, may be taken by some to be a guarantee of their safety for man. However, this evidence alone is insufficient to warrant the unrestricted use of materials which have not been properly examined in the laboratory. Some chronic toxic effects, including carcinogenesis, are insidious, and detecting cause and effect relationships is no easy matter. The dramatic story of aflatoxin has taught us, or reminded us, of another thing. A knowledge of the toxicology of only the major constituents of a mixture is not enough. Biologically, significant toxicity may be attributable to a substance present in only a trace amount, or to a substance which is present under some, but not other, circumstances. It seems then that if continued use is to be made of essential oils, particularly in food, or as aerosol sprays, sooner or later a systematic study will have to be made of the pharmacology and chronic toxicity of all their constituents. One would not wish to exaggerate the urgency of this need. No doubt there are many other insufficiently investigated environmental factors which merit greater concern. On the other hand, in the event of man suddenly increasing his degree of exposure to a traditional environmental factor, he ceases to be "protected" by the "safety" guarantee provided by the tradition. For this reason we are bound to take a serious view, for example, of the increased use in recent years of safrole in root beer.

In general it is right that we should spend a great deal of energy in making sure that in the course of advancing in the fields of food and cosmetics technology we do not add new carcinogens or other toxic agents to the environment. However, it should be emphasized that if all our energies are spent in this way, and even if we were 100% successful in these endeavours, we should do nothing to lower the incidence of cancer or other forms of intoxication which are attributable to factors already present in the environment. In other words, if we wish to reduce the present incidence of cancer in man we must examine most carefully all the factors which go to make up his traditional environment. In this connexion a careful and thorough study of the essential oils is long overdue.

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*Née Peirce.

The Complexing Action of Eugenol on Sound Dentin

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SYNOPSIS IN INTERLINGUA

LE ACTION COMPLEXATORI DE EUGENOL SUPER DENTINA NORMAL.—Eugenol a oxydo de zinc (EOZ) in applicationes clinic mollifica le dentina. Eugenol o aqua esseva addite in un proportion de 10:1 per peso a dentina in sectiones de 150 μ. Le concentration de Ca in eugenol accresceva a 21,5 mg per 100 ml intra 6 septimanas. In le systema de controlo a aqua le correspondente valor esseva 3 mg per 100 ml. Mixturas de EOZ esseva applicate a sectiones de dentina e le blocos de EOZ esseva tincturate pro Ca^{++}. Le acceptation de Ca^{++} esseva positive e le controlo esseva negative. Iste datos demonstra le capacitate de eugenol e de EOZ de remover Ca^{++} ab dentina, possibilemente con le formation de un chelato.

The past several decades have brought forth numerous investigations of the properties and uses of zinc oxide-eugenol (ZOE) mixtures. Of particular interest to those studying these mixtures has been the mechanism of the setting process. In 1955, Copeland and coworkers,[1] using x-ray diffraction data and infrared absorption spectrums, demonstrated that zinc eugenolate $Zn(C_{10}H_{11}O_2)_2$, a metallo-pyrocatechol complex or chelate, was formed during the setting process. They also extracted the excess or unreacted eugenol from the set mix with chloroform. Approximately 65 per cent of the eugenol was extractable with chloroform from a zinc oxide and eugenol mixture with extremely fine-particle zinc oxide, and approximately 85 per cent of the eugenol was similarly extractable from a zinc oxide and eugenol mixture using commercial grade zinc oxide of moderately large particle size.

Since eugenol can form a chelate with zinc ions, it was speculated that it could form a chelate with calcium ions. The known properties of calcium, namely its coordination number and ionic radius, would not be prohibitive to the proposed calcium eugenolate. Martell and Calvin[2] have shown that such replacement of zinc by calcium does occur in the ethylenediamine complexes.

After the clinical application of zinc oxide and eugenol on sound dentin in this laboratory, the underlying dentin was observed to be slightly to moderately softer than prior to the application. In light of the clinical observation and the reports on the chemistry of the setting mechanism, it was thought that the action of both eugenol and a mixture of zinc oxide and eugenol on sound dentin should be investigated.

Materials and Methods

SELECTION OF DENTIN SAMPLES.—Thirty-seven third permanent molars, completely formed and without carious lesions or structural anomalies, were collected from the Department of Oral Surgery, College of Dentistry, University of Nebraska. Immediately after extraction, each tooth was placed in a 4 per cent formol. A sectioning machine† with a water spray was used to separate the roots of the teeth, and the enamel was removed under a continuous spray of water.

Twenty-five crowns were sectioned. Two satisfactory sections, 150 μ thick, anatomically located between the dentinoenamel junction and the pulp chamber, were obtained from each of the 25 crowns. On each section, a rectangle 6.7 mm. by 6.4 mm. was drawn. The dentin peripheral to the inscribed rectangle was removed from each section. The two sections from each crown were weighed as a unit and placed in 4 per

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† Hamco Thin Sectioning Machine, Hamco Machines Inc., Rochester, N.Y.

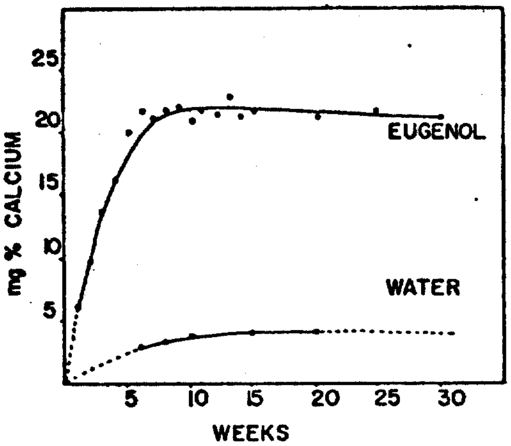

Fig. 1.—The effect of time on the removal of calcium from dentin slices, 70 μ thick, with eugenol and with water. Liquid-solid ratio was 10:1 by weight.

cent formol in separate containers. The two slices from each crown weighed approximately 150 mg.

The remaining 12 crowns were sectioned in the same way; however, the slices were 70 μ thick, resulting in three or four sections per crown. The sections were not inscribed with a rectangle but were stored in formol in their original containers after being weighed as a unit.

Action of Eugenol on Dentin.—Eugenol was added to 18 pairs and water was added to seven pairs of dentin rectangles. The proportion of liquid to dentin was 10:1 by weight. The samples were placed in a vibrating apparatus from which samples were removed at periodic intervals. The calcium content of the liquid phase from each sample was determined by the Ferro-Ham method.[3] The method was modified by adding 0.25 ml. of a 1:1 alcohol-ether solution to 1.00 ml. of eugenol to increase the solubility of the latter. Preliminary studies demonstrated that this modification did not alter the quality of the method. Calcium determinations were also performed on water and eugenol contamination controls at the end of the experimental period. All calcium determinations were run in triplicate, using appropriate calcium standard solutions for verification.

Histologic evaluation.—Three groups of 70-μ sections were treated as follows: 13 sections were placed in eugenol, ten sections in distilled water and another ten sections in a 5 per cent solution of disodium ethylenediaminetetraacetic acid (EDTA). The pH was 8.0. The proportions were by weight as above. All of the samples were vibrated for 3 weeks, except for three of the eugenol-treated samples that were vibrated for three additional weeks. All sections were treated with the von Kossa staining technic[4] for the identification of the relative amounts of calcium remaining in the various dentin sections.

Action of Zinc Oxide-Eugenol on Dentin.—Zinc oxide-eugenol mixtures were prepared by adding 2 drops of eugenol from a 1-ml. transfer pipette to 250 mg. of zinc oxide. Mixtures were applied to seven 70-μ dentin sections, placed in plastic containers and incubated at 37° C. for 6 weeks.

The ZOE mixtures were separated from the dentin sections in toto and analyzed for calcium by staining the ZOE block by the Glycoxal* bis (2-hydroxyanil) method (GBHA method) of Kashiwa and Atkinson.[5] The intensity of the stain on the surface of the ZOE block that was adjacent to the dentin was then compared with the opposite surface, which was not exposed to the tooth tissue.

Results

The amount of calcium removed from the dentin samples by eugenol in 30 weeks is shown (Fig. 1). The calcium values represent an average of three determinations. There is a large increase in the calcium concentration of eugenol during the first 6 weeks, rising from 6 mg./100 ml. to approximately 22 mg./100 ml. From the sixth week to the end of the experimental period, the values remain essentially constant, the average being 21.5 mg./100 ml. The lowest reading during this period was 20.9 mg./100 ml., and the highest was 23.0 mg./100 ml.

The amount of calcium removed from the dentin samples by distilled water during a 20-week period is also shown (Fig. 1). During the first 4 weeks, the calcium concentration was not detectable; from 6 to 20 weeks, the value increased from 2.8 mg./100 ml. to 4.1 mg./100 ml. A 30-week control was not available; however, it is expected to be close to 4.0 mg./100 ml. as the trend of the values from 8 to 20 weeks ap-

* Glyco Chemicals, Division of Chas. L. Huisking & Co., New York.

pears to be leveling off. The calcium contamination controls measured at the end of the experimental period were negative.

The dentin samples treated with water, eugenol, and EDTA, respectively, for 3 weeks were stained with von Kossa's stain. These were rated from 1 to 6 according to the relative staining intensity. The water controls had a relative staining intensity of 6, indicating a considerable quantity of calcium staining salts. The dentin samples exposed to eugenol for 3 weeks had an average value of 4, and the three sections exposed to eugenol for 6 weeks had an average value of 3, indicating less calcium content. The sections of dentin treated with EDTA were devoid of any stain, indicating that the calcium was completely removed.

The ZOE mixtures that were separated in toto from the dentin sections were stained by the GBHA method for the presence of calcium ions. The surface adjacent to the dentin stained red, indicating the presence of calcium ions, whereas the surfaces not exposed to dentin were devoid of any stain.

Discussion

As shown (Fig. 1), eugenol has the ability to initially remove calcium from dentin at a rather rapid rate. The values from the sixth week to the end of the experimental period are quite stable in spite of the large excess of eugenol. A diffusion barrier might possibly exist at the eugenol-dentin interface. This could be evaluated experimentally by increasing the revolutions per minute of the mixing device: an increase of revolutions per minute should lead to an increase in the amount of calcium in the eugenol phase.

During the process of standardizing the experimental procedures, different types of eugenol were evaluated. It was found that the chelating ability of eugenol varied with the different types of eugenol. It also appeared, that the older eugenol was less effective in removing calcium ions from dentin. Because of the shortage of samples, this was not established. The eugenol finally selected exhibited good chelating ability.

The reliability of von Kossa's stain was tested by means of partially decalcifying several tooth sections and comparing the amount of calcium removed from the specimens with the intensity of the stain. It was found that the intensity of the stain diminished proportionately with an increasing amount of decalcification. In several sections that were completely decalcified, the stain was completely absent. The results obtained from the dentin samples treated with von Kossa's stain indicate that eugenol is capable of removing more calcium from the dentin sections than water. The chelating properties of eugenol are significantly more limited than the chelating ability of EDTA when both chelators are used in the same proportions. It can be speculated that eugenol would eventually decalcify the tooth sections completely if changed periodically.

Brauer and associates[1] showed through x-ray diffraction and infrared studies that not all of the eugenol entered into the formation of the zinc eugenolate. They also concluded that 85 per cent of the eugenol can be recovered after the setting of ZOE mixtures. The results of this study indicate that the eugenol that does not enter in the setting mechanism of the ZOE mixture is capable of chelating calcium ions from dentin. Because of the difficulty involved in completely separating the ZOE mixtures from dentin, it was not possible to make quantitative determinations of the amount of calcium removed from the dentin sections.

The failure of ZOE to produce a calcified bridge of secondary dentin at the site of pulp exposure has been reported by several investigators.[6, 7] It may be possible that the complexing action of eugenol with calcium prevents the mineralization of the collagen matrix formed beneath the site of pulp exposure, thus preventing the formation of a dentin bridge.

Although the laboratory procedures were not intended to duplicate the clinical application of ZOE, it is believed that the softening of sound dentin beneath a ZOE mixture is explained on the basis of the eugenol's ability to complex with calcium.

Summary

The action of eugenol and a mixture of zinc oxide and eugenol on sound dentin was studied by chemical and histologic means. Both demonstrated the ability of eugenol and ZOE mixtures to progressively remove calcium from dentin. The softening of sound dentin beneath clinically applied ZOE mixtures was thought to be a result of the complexing action of eugenol with calcium.

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Protistacidal (Protozoacidal) Action of Essential Oils and of Some Organic Compounds

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With the purpose of investigating new antitrichomonadal agents, at the Kharkov Scientific-Research Chemico-Pharmaceutical Institute, studies were carried out on the protistacidal action of a number of essential oils and of other organic substances of vegetative origin.

In the capacity of a testing object, use was made of Paramecium caudatum and of Trichomanas vaginalis. A loop of the substance under study, in a suitable dilution, was mixed on a coverglass with a loop of Infusoria culture or with a loop of trichomonadal suspension in a physiological solution, obtained from patients ill with trichomonadal colpitis. The protistacidal action was studied in a suspended drop under a microscope. The authors made a study of essential oils -- geranium, clove, eucalyptus, dill, lemon, lavender, cow parsnip, poplar, tansy, and wormwood (the four last-mentioned oils were obtained by senior scientific worker Z. V. Sovaya of the KhNIKhFI (Kharkov Scientific-Research Chemico-Pharmaceutical Institute). In addition to this, a study was made of organic compounds that represent the components of the essential oils: aldehydes -- anisal, benzoic, cinnamic, para-butylbenzoic; aliphatic saturated alcohol -- octanol; phenols -- para-butylphenol, methyl-tertiary butylphenol, methylethylpropylphenol, para-isoheptylphenol, ortho-propenylphenol, para-isopropylphenol, para-isoamylphenol, isoeugenol, cyclo-geraniol; mono-cyclic terpene -- limonene.

Of the essential oils, the strongest action on the Infusoria was manifested by the geranium, lemon and clove oils, which brought about the destruction of the Protozoa in dilutions of 1 . 10^minus 5 - 1 . 10^minus 6. The cowparsnip, poplar, tansy, wormwood, and eucalyptus oils acted in dilutions of 5 . $10^{minus\ 4}$. Among the tested chemical compounds, the most effective turned out to be the phenols, which brought about the destruction of Infusoria in dilutions of 5 . $10^{minus\ 4}$ - 5 . $10^{minus\ 5}$ in less than 1 minute, and in dilutions of 1 . $10^{minus\ 6}$, after a 1 - 4 minute action. The dissolution of the substances being tested, in a 2.5% solution of glycerin, noticeably intensified their protistacidal activity.

In carrying out tests on trichomonads it was established that the strongest antitrichomonadal properties were possesses by para-butylphenol and methyl-tertiary butylphenol, which acted in dilutions of 1 . $10^{minus\ 5}$ - 1 . $10^{minus\ 6}$. The cow parsnip, poplar, geranium, and clove oils, and also, cinnamic aldehyde, isoeugenol and cyclogeraniol, in dilutions of 1 : 200, brought about the destruction of trichomonads in less than 1 - 2 minutes. Other essential oils, in this dilution, manifested an effect after 3 - 10 minutes of action.

The character of the action, on the part of the tested substances, on trichomonads, was, in general, monotypic: the forward movements of the Protozoa were discontinued; there were merely noted the oscillatory and rotational movements; after that, the pear-shaped body of the trichomonad became rounded, turned stationary, and the cell died.

A determination of the toxicity of essential oils and of other organic compounds, in experiments on white mice, upon administering through a probe into the stomach, indicated that the Dl_{50} of para-butylphenol, methyl-tertiary butylphenol, isoeugenol, cyclogeraniol, and cinnamic aldehyde comprises 10 - 20 milligrams for a mouse (with a weight of 16 - 18 grams). For essential oils, the magnitude of DL_{50} fluctuated within the range of 3 -5 milligrams of a 1% emulsion of cow parsnip, poplar, and tansey oils and of cinnamic aldehyde; with a 7-day administration into the conjunctival eye sac of a rabbit, they did not manifest any local irritating action.

Along with the antitrichomonadal properties, the tested substances possess a strong antibacterial action, which inhibits the Gram-positive and Gram-negative microflora.

The results of the investigation yield a basis for recommending parabutylphenol, octanol, cinnamic aldehyde, cyclogeraniol, isoeugenol, and also, cow parsnip. poplar, geranium and clove essentials oils for clinical testing in the capacity of antitrichomonadal agents.

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CONCEPT OF THE MUCOUS BARRIER AND ITS SIGNIFICANCE*

II. Changes in the Gastric Mucosa Produced by the Local Actions of Spices and Other Irritative Agents

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INTRODUCTION

Although considerable progress has been made concerning the functions of the parietal and peptic cells of the stomach, the knowledge of the anatomy and physiology of the gastric mucous epithelium is quite meager. This is more surprising when one considers that for a long time mucus has been known to play a protective role on the gastric mucosa. To what extent this protection is effective and how it works is not well known, in spite of the fact that diseases as formidable as peptic ulcer and carcinoma of the stomach are encountered with such relative frequency. Therefore, this study was devoted to investigate the changes produced in the gastric mucosa by the action of spices as well as some other irritative agents. This was done in a rather detailed manner and comparative studies of what happens in colonic and small bowel mucosa were also carried out.

REVIEW OF THE LITERATURE

Mucus has been found to be the usual response of the gastric mucosa when subjected to a wide variety of chemical or mechanical traumata[4, 18, 22, 25, 28, 36, 37, 52, 54]. The increased production of mucus as a response to irritative agents has been considered for a long time a protective mechanism. Bernard pointed it out in 1856[11] and since many authors have mentioned it. Wolf and Wolff[55] noticed that when the mucus is wiped away from the gastric mucosa, this structure is less resistant to any kind of irritant, and engorgement, bleeding and ulceration ensued quite rapidly. Similar response of the mucosa was observed by Whitlow[53]. It has been found that mucin inhibits peptic digestion and also restrains the acid-peptic juice from coming into direct contact with the cells of the gastric mucosa[5, 17, 27, 46, 53].

It has also been observed that mucus secretion is alkaline[3, 13, 14, 33, 35, 50, 55] and Hollander and Lauber have obtained a mucous secretion with a pH as

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high as 8.6[31]. The possibility that direct neutralization of the acid of the stomach can be effected by the mucus has been considered by many[13, 14, 16, 17].

Wolf and Wolff[51] believe that "mucus is chiefly, if not wholly, responsible for neutralizing the product of the parietal cells". Concerning the histology of the mucus producing cells, complete agreement is lacking, not only as to the identity of the different types of cells, but also as to the nature of their secretion. Lim studied the gastric mucosa of cats, dogs, rabbits and humans. He concluded that mucus is produced by the mucoid cells of the tubular glands, the same cells that Bensley[10] calls mucous neck cells. This last author believes that the pyloric glands, the cardiac glands and the mucous neck cells belong to the same type. Ivy and Oyama[35] concluded from their studies that the secretion of the pyloric glands is simply mucus. Webster and Komarov[52] stated that there are two different types of mucin secreted by the stomach, one originated by the surface epithelium, visible as threads and clumps in the gastric secretion, and another obtained from filtered acid gastric juice, called by them dissolved mucin. They suggest that the latter is secreted by cells of the tubular gastric glands (mucous neck cells?).

The relationship of the gastric mucous secretion to the pathogenesis of peptic ulcer has been scrutinized, as one would suspect. Kaufmann[37] in 1908, stated that the pain in patients with hyperacidity was due to lack of mucus. Hurst[32] said in 1933, that a hypersthenic stomach is capable of secreting very little mucus compared with the hyposthenic stomach, and therefore the protection of the mucus in those cases, is absent. Recently Meyer et al.[42] reported that pure lysozyme washed the mucous covering off the mucosa of Pavlov pouches. They also reported that the lysozyme content in the gastric juice of normal individuals was less than in individuals with duodenal ulcer.

Studies on the regeneration of the gastric epithelium after damage to the superficial structures have been made by Hollander and co-workers[28, 29, 30] and Rhoda Grant[26]. Ferguson[19] among others has studied regeneration when the whole mucosa is destroyed.

A. Effect of Spices on the Gastric Mucosa of Dogs when Acting for Short Periods of Time

Method: A group of mongrel dogs was laparotomized under anesthesia with intravenous Nembutal and the stomach was opened, maintaining insofar as possible an intact blood supply to the viscus. Pledgets containing the spice were then placed on several areas of the gastric mucosa and the effect noted. Suspension of 1 per cent of the spice was used. Pledgets soaked with physiological saline were used as controls. There were four experiments with each spice and observations were made over a three-hour period.

Results: The following spices were tested: Celery salt, cinnamon, cloves, mustard, nutmeg, paprika, pepper and sage. The pattern of response was the same in all cases. On opening the stomach, a thin layer of glistening, transparent mucus covering the mucosa was seen. After the pledgets were applied, the secreti onof mucus was visibly increased; later, it became turbid and finally changed to an opaque white-gray.

When mustard was applied, and to a lesser extent when paprika, pepper, and cinnamon were used, there appeared to be a progressive edema of the mucosa and after a three-hour period, this became obvious when compared with the control areas. The changes induced by the other spices tested were negligible. No attempt was made at any time to remove the protective layer of mucus covering the mucosa, and in that condition no bleeding or erythema of this structure was observed.

Patches of gastric mucosa were transplanted to the abdominal wall, as described by Florey and Harding[21]. A continuous drip of a 1 per cent suspension of the spice in isotonic saline was applied to the transplant. The response was the same as that observed when pledgets were applied to the gastric mucosa.

B. Effect of Spices and other Irritants on the Gastric Mucosa, Small Bowel and Colon

Method: Several groups of experiments were carried out. Dogs and cats were used. The animals were anesthetized with Nembutal in doses of 15 milligrams per pound.

Group I: The gastric mucous cells were stimulated by placing various substances into the stomach after having ligated the esophageal and pyloric orifices. Mustard oil and clove oil in concentrations of from 0.15 to 2 per cent (in corn oil), and eugenol in aqueous or oily solutions up to 10 per cent were used. The animals were sacrificed within 3 to 10 hours after the injection of the irritant.

Group II: The animals were injected with the irritant as described above and after the period of exposure to the irritant, they were re-opened, the stomach was emptied, the ligatures were removed and the wound was closed. The animals were allowed to recuperate and two or three weeks later they were subjected to the same procedure (stimulation and recuperation).

Biopsies of the stomach were taken before and after the period of irritation in both groups. In group II, biopsies were also taken 24 and 48 hours after the period of stimulation was stopped. The biopsies were taken by doing a laparotomy, cutting through the wall of the stomach until the mucosa was reached and then a small portion of mucosa was removed and immediately fixed in 10 per cent formalin.

Group III: With the purposes of comparing the results of stimulation in the gastric mucosa with what happens in colonic and small bowel mucosa, injection of the mentioned irritants was done on closed loops of colon and small bowel.

A loop of colon, about 6 cm. distal to the ileo-cecal valve, and from 6 to

10 cm. in length was used. The fecal contents were removed, a biopsy of the mucosa was obtained, the ends of the loop were tied and the irritant injected with a 19 gauge needle. A filling of the loop without pressure, conditioned the amount of irritant injected. This procedure was very much like that described by Florey and Webb[22]. It was performed seven times, the period of stimulation varying from 3 to 10 hours.

Loops of small bowel, about 10 cms. in length, were likewise ligated at both ends, biopsies of the mucosa were taken and thereafter the irritant was in-

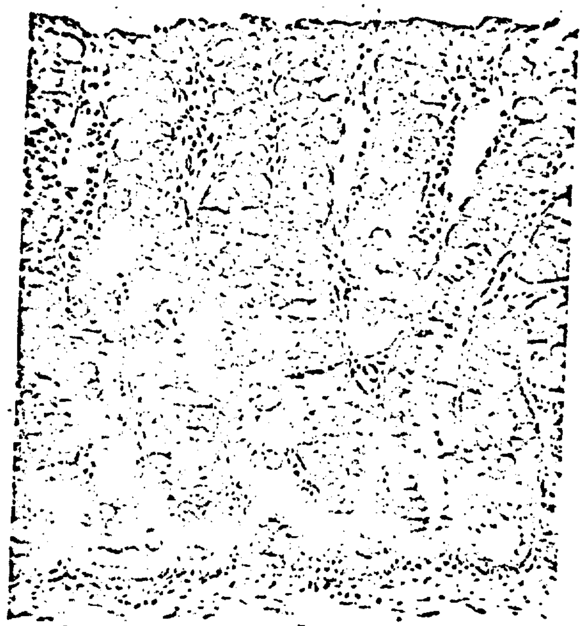

FIG. 1. Cat No. 2. Colonic mucosa Stain: (this and all the following sections shown were stained with Mayer's Mucicarmin).

jected. The procedure was performed 16 times, for periods varying from 3 to 10 hours.

Group IV: In this series, cervical esophagostomy and duodenostomy were performed on dogs. Cannulas were introduced through both stomata, biopsies taken, and the wounds closed. A continuous drip, using 0.66% suspension of the spice in normal saline, was then started. These animals were kept under Nembutal anesthesia until they died or were sacrificed. Adequate infusions of dextrose and saline solutions were given, and urinary retention was avoided by the use of an indwelling catheter. The animals were kept warm and were turned from side to side every 2 or 3 hours day and night to prevent pulmonary stasis. The experiments lasted from 3 to 62 hours. A total of 45 animals were used. Whenever possible, before the experiment was started and at different periods thereafter, biopsies of the esophagus, fundus, antrum, duodenum, small bowel and colon were taken. The specimens were fixed in 10 per cent formalin and stained with hematoxylin-eosin and Mayer's muci-carmin.

Results: (a) Colon. Concentrations of the stimulants (mustard oil and clove oil) from 0.5 to 2 per cent for periods ranging from 5 to 8 hours produced the following changes: macroscopically, the loops were found to be considerably

FIG. 2. Cat No. 2. Colonic mucosa after stimulation with mustard oil in corn oil (120 drops per 100 cc.) for 6:15 hours. Notice the striking change of the goblet cells. Now, the epithelium is flattened and there is obvious widening of the crypts.

distended and filled with a gelatinous, clear mucus. The mucosa was red and hemorrhagic and the wall edematous. Histologically, the most striking feature was the emptying of the goblet cells. Normally the goblet cells have a hyperchromatic nucleus, triangular or crescentic in shape that is displaced to the basal portion of the cell, the rest of which is a globular mass of mucus.

After stimulation to the point of depletion was produced, the goblet cells became cuboidal, with a pale, round nucleus occupying the center of the cells and with no apparent mucus. The epithelium at this stage looked as if it were formed by a unique type of cells: flat, cuboidal and identical in appearance

(Figs. 1 and 2). (These findings suggest that maybe the difference in morphology which one sees in the normal colon represents only the same type of cells at different functional stages. In this regard, Bensley[10] states that "It is obvious and it is indeed true, that cells which are strictly homologous with each other and concerned with the same sort of activity, by reason of the different levels at which equilibrium is attained with reference to the rates of the various phases in their presecretory processes, may seem, under the microscope, to

FIG. 3. Dog. No. 125. Colonic mucosa after stimulation with mustard oil in corn oil (40 drops in 100 cc.), for 6 hours. Notice the destruction of the epithelium in the top, depletion of the goblet cells in some areas and flattening of the epithelium.

be very different from each other".) The depletion obtained in the mucous cells varied from partial to complete in each cell and in the whole epithelium. Differences in degree of depletion were observed in different areas of the same loop and in different animals. On the whole, the degree of depletion obtained was roughly proportional to the strength and duration of the stimulus.

Dilation of the lumen of the crypts was also seen, especially apparent in the lowermost portion. An increased number of mitosis was also noticeable.

When the stimulant was applied beyond the stage of depletion, either by increasing its strength or by increasing the time of application, necrosis took place. In the sequence of events observed, several simultaneous phenomena took place: edema, hyperemia, extravasation of red blood cells and leucocytic infiltration. Subsequently, the epithelium at the top of the crypts was shed and the cells of the stroma burst and spread over the mucosal surface (Fig. 3). At this stage, although definite necrosis was visible on the surface, no sign of depletion was observed in the goblet cells at the bottom of the crypts. The layer of mucus and cellular debris formed an "umbrella", that, for a while, seemed to preserve the cells of the crypts from coming in close contact with the irritative

FIG. 4. Cat No. 5. Small bowel mucosa. Notice the distribution of the goblet cells in the villi.

agent. If the stimulation persisted, the necrosis progressed from the surface down, more cells were destroyed (hence less mucus was produced) and finally the whole mucosa underwent a process of disintegration.

Loops of colon tied at both ends for similar periods of time, without injection of any irritative substance, showed a moderate increase in mucus production with no obvious histological change.

The results obtained were very much of the same nature as those obtained by Florey and Webb[22] in the colon of the cat. The techniques in both cases were quite similar.

(b) Small Bowel: Injection of very dilute concentrations of mustard oil, less than 5 drops per 100 cc., produced a diminution in the number of goblet cells. Counts of the cells before and after stimulation showed the decrease to be real. (Figs. 4 and 5). Some instances were found in which the base of the crypts was dilated and flattening of the surface epithelial layer was observed. Although these findings were not frequent, they were definite and gave the impression that the process of depletion of the goblet cells is similar to that observed in the colon, although more difficult to obtain, since the small bowel

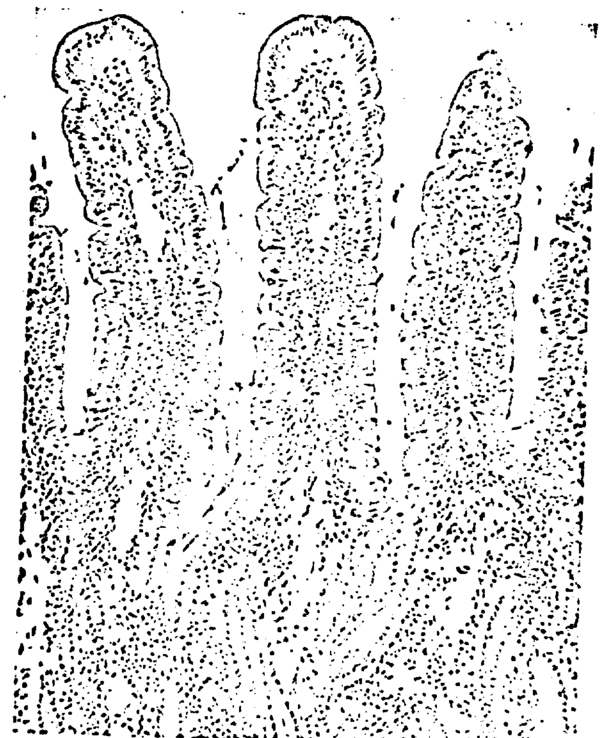

FIG. 5. Cat No. 5. Small bowel mucosa after 4:5 hours of stimulation with mustard oil (1 drop) in corn oil (30 cc.) Notice the disappearance of the goblet cells. There was also some flattening of the epithelium of the crypts.

is much more sensitive to the action of the irritant and becomes necrotic quite readily.

The process of necrosis followed the same pattern outlined for the colon. It was observed when concentrations of the irritants as low as 10 drops per 100 cc. of the mustard or clove oil in corn oil were used for periods of 7 hours or more. These results are similar to those obtained by Florey and Webb[22].

(c) Stomach: In this study, mild stimulation of the stomach was produced using 0.66 per cent suspension of the spice in saline. The stimulus was applied as a continuous drip for long periods of time (up to 62 hours). In those animals no examination of the inside of the stomach was possible until after the animal was sacrificed, but biopsies of the mucosa were taken at different periods during the experiment. The macroscopic appearance of the mucosa when the stomach was finally opened varied from normal to that in which there was edema congestion and hemorrhage. The latter was only noted at the late stages of stimulation (over 50 hours). Before that time, the mucosa looked surprisingly "healthy", except for the presence of edema. Mucus was noticed as coming

FIG. 6. Dog 139. Mucosa after stimulation for 6 hours with 10% eugenol in water. This shows a stage in which the tops of the epithelial layer have been destroyed. Notice the deeply stained mucous neck cells, showing some evidence of depletion. Marked hyperemia present.

out in the washings in the form of white-gray clumps. Every time the stomachs were opened much mucus was found to adhere tenaciously to the mucosa sometimes appearing transparent and glistening, sometimes appearing coagulated, turbid and perhaps flecked with blood. Since the spice was dripped continuously, the washings collected were a mixture of gastric secretion and suspension of spice. Determinations of the mucin content of these liquids were made and were found to be elevated, as compared with the values in normal gastric juice.

Sections taken at different intervals from 1 to 62 hours showed remarkably is urged to investigate its copyright status.

little change. Depletion of the surface epithelial cells was not observed. At the beginning of the stimulation the cells were overactive or, at least, increased production of mucus was noticed. Later on, the histological picture varied very little as far as the epithelium was concerned although hyperemia, migration of leukocytes, and estravasation of red blood cells were noticed.

The mucous neck cells, which occupy about the upper third of the mucosal layer in both the cat and dog showed a little more change. These cells usually

FIG. 7. Cat No. 12. Gastric mucosa (fundus) after stimulation for 12 hours with 10% eugenol in corn oil. The tops of the interfoveolar spaces have shed off the mucosa and remain only attached with mucus. The continuity with the epithelial layer is beautifully shown.

present a dark hyperchromatic nucleus, crescentic in shape and displaced towards the base. The rest of the cell is stained pink-red by the muci-carmin. After stimulation the protoplasm showed a decrease of its staining property, the nucleus took a more or less ovoid shape, became clearer and moved towards the center of the cell. These changes were found later in the process of stimulation, starting perhaps about 40 hours after application of the irritant.

When strong stimulants were used, (mustard oil, clove oil and eugenol in concentrations of 2 to 10 per cent) the process of necrosis and regeneration was more thoroughly studied. The irritant was placed in the stomach with both cardia and pylorus ligated, and the changes produced were observed at different intervals. Macroscopically, the amount of mucus obtained was always striking. In several instances, a gelatinous mass was found to fill the stomach completely. This mass formed a perfect cast of the viscus, and when left alone, shrank down markedly exuding a sero-sanguineous fluid.

Microscopically, deepening of the foveolas was first noticed, with narrowing of the interfoveolar spaces. This was soon followed by hyperemia, leucocytic

FIG. 8. Dog 139. Gastric mucosa 14 days after stimulation for 6 hours with 10% eugenol in water.

infiltration and extravasation of erythrocytes, together with progressive edema. Sometime later, the most superficial portions of epithelium became detached from the body of the mucosa, leaving only the deepest portions of the foveolae (Fig. 6). When irritation ceased at this stage, the continuity of the surface epithelium was restored completely in from 24 to 48 hours. When the irritative process was not stopped, progressive destruction of the mucosa ensued, the cellular elements burst and disintegrated and complete necrosis occurred.

It is interesting to mention that while the gastric mucosa is still histologically normal after a period of several hours of irritation, the esophagus exposed to similar trauma presents a gross appearance of heavy mucosal hemorrhage with microscopic exfoliation of the epithelium (either localized or extensive), and marked leucocytic infiltration around the desquamated areas[15].

In some cases, after a period of acute irritation the animal was allowed to recuperate and the procedure of irritation was repeated subsequently. Biopsies of the mucosa taken before the second period of irritation was started, showed changes consisting of cystic areas scattered in the body of the mucosa, areas of degeneration and a ragged contour of the epithelial surface as observed in the section. Some foveolae were deeper than others instead of exhibiting the usual fairly even depth (Fig. 8). The mucosa under these circumstances, proved to be more susceptible to the action of the irritant than in the initial period. Necrosis ensued more quickly and the ability of the cells to take the carmin stain was markedly decreased. Regeneration, too, was impaired. In comparison with the healing process of the first period, more irregular epithelium covered the defects the second time, more cystic areas and more areas of degeneration were observed.

One striking feature in the regenerative process is the appearance of the epithelial cells. These elements, usually cylindrical, change in shape and acquire the appearance of a squamous cell, completely flat and with very scanty mucin if at all. These cells cover the denuded areas at first, and as soon as continuity is restored, they progressivel change to cuboidal and later to cylindrical shape again, regaining their mucous content.

DISCUSSION

In this study, when comparison was made between the results obtained on persistent stimulation of colon, small bowel and stomach, three categories of changes were noticed. The first covers the changes of the mucosa from normal to necrosis. The second covers the changes observed in the mucous-secreting cells alone. The third covers the regenerative changes observed in the epithelium of the gastric mucosa following its partial destruction by the irritative agent.

The changes from normal to necrosis were essentially the same in stomach, small bowel and colon. There were quantitative differences inasmuch as the stomach is, by far more resistant to the action of irritants. The congestion observed may play an important part in the secretory activity of the mucous cells, since Florey[20] observed that the passage of fluid from the blood stream was necessary to effect the discharge of the concentrated mucin contained in the goblet cells of the colon.

Sometimes stimulation insufficient to produce necrosis of the gastric mucosa produced localized ulceration of the small segment of duodenum exposed to the irritative agent. This event suggests that the duodenal mucosa is more sensitive than the gastric mucosa to the same type of irritant. It also suggests since the secretion produced by eugenol is frankly alkaline, that ulceration of the upper gastro-intestinal tract may take place in the presence of little acid.

When the depletion of the mucous cells was studied alone, very interesting observations were made. Stomach, colon and small bowel responded in a different manner. The depletion in the colon was easily obtained. In the small bowel, before complete depletion of the goblet cells was seen, necrosis was already taking place and, therefore, complete exhaustion was not observed. In the stomach the resistance of the cells to the action of the irritants was much greater than in the colon. When the stimulating substance was applied the surface epithelial cells entered into a phase of active secretion and apparently continued to secrete for long periods of time without microscopical evidence of fatigue. The neck cells as well as the pyloric glands showed an equivalent resistance, although the neck cells indicated evidence of beginning depletion about 40 hours after the stimulation was started. The production of massive amounts of mucus with no histological evidence of depletion of the cells, that was also observed by Florey and Webb[22] speaks for a remarkable resistance of the epithelial layer, as compared to that of the colon and small bowel. An explanation can be ventured linking the described phenomena with the observations of others[9, 19, 26, 28, 29, 30, 44].

In the first place, the gastric mucosa should be imagined as being somewhat different than the way it is usually described. Instead of conceiving it as a surface with scattered depressions, called foveolae, it can be visualized as a surface with a multitude of protrusions in it, very much like intestinal villi, but closely packed together. Those protrusions are nothing but the interfoveolar spaces and could be called "gastric villi". With this image in mind, let us follow the response of the mucosa to any type of irritative process. At first, there is increased production of mucus that contains very few desquamated cells. Later, there is narrowing of the stems of the "villi" and subsequently shedding of its upper portion. This means more mucus macroscopically, more desquamated cells in it microscopically.

If the irritative agent stops at this point, immediate regeneration takes place. Obliteration of the denuded area on each one of the "villi" is effected by a new layer of epithelial cells that in all probability come from the zone at the bottom of the foveola[10]. The process of regeneration can be compared to what happens in the skin after cutting a graft: regeneration takes place from epithelium around skin appendages and the tips of the dermal papillae. If the entire thickness of the skin is destroyed, regeneration takes place from the margin of the wound. Similarly, if the entire thickness of gastric mucosa is destroyed, regeneration takes place from the margins of the defect[20]. Every pathologist has observed this in healing peptic ulcers.

Each one of the denuded areas is very small, since thinning of the interfoveolar spaces may leave exposed only a very short segment that can be covered by a few cells. This mechanism goes on, very likely, under normal conditions, developing at a rate in accord with the requirements imposed upon the mucosa, slowing down or stopping completely in a protected stomach or speeding up when acute and violent irritation occurs[26]. Actually, several protective mechanisms, or better perhaps, several aspects of the same protective mechanism add up to give the gastric mucosa an unexcelled protection. The mucus contained in the cells can be discharged into the foveolae upon demand. This mucus adheres tenaciously to the mucosal epithelium, in such a way that very little direct contact can be effected between the cells and anything in the gastric cavity. The role of the mucus in neutralizing the action of acid and pepsin has been mentioned. The increased secretion of mucus under "alarm" conditions has also been considered. All this, together with the shedding of the epithelium as described, suggests a very effective protection. Farrell[18], Ivy[31] and Hollander et al[29] have called "mucous barrier" the protective mechanism of epithelial cells and mucus together. The concept of the defensive mechanism, as described, adds to a more significant and important meaning of this gastric "mucous barrier". Its protection is quantitatively, and perhaps also qualitatively, superior to that present in any other region of the gastro-intestinal tract. A brief comparison of the stomach with the rest of the gastro-intestinal canal demonstrates this different type of protection.

In the esophagus, there are some scattered mucous glands, few in number, and the protection they can give to the structure, of necessity is poor. A continuous drip of gastric juice or a solution of mustard and clove oil into the esophagus and stomach produces marked changes in the former when the latter is still grossly intact[46].

Beyond the stomach there are goblet cells in the intestine and colon, but these cells are not as resistant as those in the stomach to the action of irritants or gastric juice.

Varco and Wangensteen[49] produced defects in the gastric wall that were kept open by means of a metallic ring. The defects were covered with omentum. At a later date, the omentum was found to be covered with a layer of gastric epithelium. This suggests a mechanism of protection in which the major role belongs to the gastric epithelial layer.

Disturbances in the protective mechanism of the "mucous-barrier" may well be responsible for such states as chronic gastritis, atrophic gastritis, etc., and may well be a preceding situation to the favorable action of carcinogenetic agents, whatever they may be. A defective function of this protective mechanism may be of primary importance too in the pathogenesis of gastric ulcer. The role of acid in the production of peptic ulcer is well known. To what extent is that true in *gastric ulcer?* The gastric mucosa resists normally the acid content of the viscus much better than the duodenum does[6,21,41,61]. On the other hand edema, as well as vascular changes have been found to abet the incidence of histamine-provoked-ulcers[7,8,24]. Perhaps then a decreased resistance of the gastric mucosa to the acid-peptic action may be, as has been suggested[6,31], a rather important condition in the production of ulceration in the stomach. Changes in the "mucous barrier" mechanism may hence, be of some importance.

One thing is evident: whatever the importance of the "mucous barrier" proves to be, the integrity of this protective mechanism should be preserved or restored in any patient with ulcer diathesis. Why then has mucin therapy given such questionable results? Ever since Fogelson's original report[23], experimental data on mucin have been contradictory[12,15,38]. Contradictory also have been the reports on the comparative concentrations of mucin in the stomach of individuals with and without ulcer[1,2,12,16,43,48]. The fact is that it is not the mucus concentration in the gastric contents that counts, but the protection that the mucous barrier gives. This means a layer of mucus in close contact with the mucus of the epithelial cells, a condition that cannot be imitated by the ingestion of any commercially prepared mucin.

How a deficiency in mucus production can be ascertained is still an open question, since mucus determination in the gastric contents is not reliable. This has been pointed out by Wolf and Wolff[56]: "The measurement did not necessarily reflect the quantity of mucus produced . . . since the material . . . may adhere to the wall of the stomach and not appear in the specimen withdrawn". This observation was confirmed in the course of these studies. The defense mechanism could theoretically be enhanced by a substance which would act exclusively or preferentially as a stimulant to the mucous-secreting cells, without stimulating the parietal cells and producing little or no irritation of the gastric mucosa. Investigations along this line might prove fruitful.

In the meantime, the possible deleterious effects which irritants produce in the gastric mucosa should be considered as of some importance.

SUMMARY AND CONCLUSIONS

1. The action of spices on the gastric mucosa was studied. It was found that after 3 hours of direct contact with the mucosa, nutmeg, sage, celery salt and cloves produced little or no change, while some edema was noticed with pepper, cinnamon, paprika and mustard.

2. Mustard and cloves, in aqueous suspension and in oil, together with eugenol, were used in experiments aimed at the depletion of the mucous-producing cells of colon, small bowel and stomach. It was found that as a whole, two categories of changes were produced in the mucosa of the organs studied.

This hypothetical division was made for the purpose of clarity. There were changes produced by stimulation of the mucous secreting cells and changes produced by irritation of the whole mucosa.

3. The changes produced by irritation varied from edema and congestion, to necrosis. The variation was roughly proportional to the strength of the stimulus used and to the period of application. The changes were quite similar in the three types of mucosa studied, although quantitative differences in response were observed. The stomach was highly resistant and the small bowel very susceptible, to the action of the irritant. The colon occupied an intermediate position, being less susceptible than the small bowel but much less resistant than the stomach.

4. The changes observed on the mucous cells varied in the different segments studied. In the colon, depletion of the goblet cells was easily obtained using concentrations of 0.5 to 2 per cent of mustard and clove oil (in corn oil) for periods ranging from 5 to 8 hours. In the small bowel, injection of very dilute concentrations of the same substance (5 drops per 150 cc.), produced a diminution in the number of goblet cells, but as a rule, necrosis ensued before complete depletion took place. Stimulation of the gastric mucosa by a 0.66 per cent suspension of spices for long periods of time (up to 60 hours) failed to produce depletion of the epithelial mucous cells. Some partial depletion was noticed in the neck cells. When strong stimulation was applied (in the form of clove oil and mustard oil in concentrations from 0.5 to 2 per cent and eugenol in concentrations up to 10 per cent for periods varying from 3 to 10 hours), a very active mucous secretion was noticed macroscopically, but no histological evidence of depletion was obtained. In cases in which necrosis ensued, areas of intact or partially necrotic epithelium did not show any depletion.

5. This apparent indefatigability of the gastric mucosa was explained on the basis of previous work and the observations described. The gastric epithelium sheds fragments of the interfoveolar areas that are replaced by new elements. In this way, the histological appearance of the mucosa may show the same stage of active function of the mucous cells, for long periods of time.

6. A concept of a defensive mechanism: "the gastric mucous barrier", was presented and discussed. It was based on the configuration of the mucosa, the protective defense afforded by the epithelial cells as well as mucus, and the ability of the epithelium to shed out fragments.

7. This concept offers an explanation to the still sought answer of why the stomach does not digest itself.

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THE EXCRETION OF THYMOL, CARVACROL, EUGENOL, AND GUAIACOL AND THEIR DISTRIBUTION IN THE ORGANISM.

Vollmer who examined the water content of lung and liverof guinea pigs after administration of expectorants reported recently that saponines, potassium.sulfoguiacolate, and extracts of Castanea vesca cause an increase in the water content of the lungs. This effect was not observed after treatment of the animals with potassium iodide, ammonium chloride, or extracts of thyme. Vollmer's findings indicate a secretolytic effect of the sapolnines, the Castanea vesca, and the potassium sulfoguaiacolate, while another mechanism has to be postulated for the explanation of the clinically established efficacy of the other two substances. Thes results agree partially with those by Gordonoff who observed by X-ray photography and fluoroscopy the change of a shadow produced by the injection of iodized oil into the respiratory tract of the rabbit, and who found a predominantly secretolytic effect with saponine preparations and a mainly secretomotoric effect for extracts of thyme. In spite of the large number of studies on expectorants performed in recent times (the work by Kochmann and Hesse should here be mentioned) comparatively little is known about the mechanism of their effect. For instance, the excretion of essential oils and some of their components by way of the respiratory tract has frequently been postulated but has never been experimentally proven. Recently, Gordonoff and Janett and Gordonoff reported on some experiments to explain the effect of thymol and extracts of thyme, one series of which should be mentioned here because of its relevance to the present study. The authors administered by mouth to three rats 500 mg thymol each, to two rabbits, 2000 mg thymol each, and to one rabbit, 5 ml extract of thyme per kg of body weight. The animals were sacrifieced after different lengths of time, and the lungs of the three rats together, those of the rabbits separately steam distilled and the distillates tested for the presence of thymol by methods described below. One normal rabbit was used as control. Gordonoff and Janett obtained positive thymol reactions with the steam distillates from the lungs of the treated animals. Their

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We have performed such tests with thymol, carvacrol, and with extracts of thyme on numerous animals and in most cases used quantitative methods. We will report on these tests in the following text. They were extended to include two similar compounds, eugenol and guaiacol. Furthermore, we did not only check the presence of these substances in the lung, but also in the blood, the musculature, liver, kidney, urine, feces, and the content of stomach and intestines, to gain exact information on the fate of the administered substances in the body. To include the excretion with the exhaled air, we have collected this over long test periods, and also tested it by the method described below. Finally, their influence on the water content of lung and liver was determined by the method outlined by Vollmer. The test substances, thymol, carvacrol, eugenol, and guaiacol are all compounds containing one free phenolic -OH group.

Varenne, Roussel, and Godefroy postulate excretion of anethole by the lung because of the characteristic odor of the exhaled air of patients treated with this substance. The same is also assumed to be true for safrole. The diazo test could not be used with these compounds, and none of the tests mentioned in the literature was sensitive enough. Quilico and Freri mention that anethole with p-nitrobenzene diazonium sulfate gives a red color in alkaline medium, a yellow color in acid. This reaction was useless for us, because p-nitrobenzene diazonium sulfate as typical indicator compound gives such colors by itself in acids and bases.

METHODS

1. Detection and Determination of Thymol.

a) Qualitative test: We determined the presence of thymol by three methods which were also used by Gordonoff and Janett. The first of these, the diazo reaction, was performed initially by us as described by these authors, by making the test solution alkaline and then adding the diazo reagent. But we found later that the reaction is far more sensitive when NaOH is added after the diazo reagent. While positive tests are obtained by the first sequence at a thymol concentration of 1:150,000, the second, more common, sequence gave a weak but still clearly visible yellow color at a concentration of only 1:800,000. The diazo reagent is prepared by treating 50 parts of 0.5% sulfanilic acid (dissolved in 1% HCl) with 1 part of a 0.5% sodium nitrite solution. The second reaction is the carbohydrate reaction. Here, 2 ml of the test solution, in this case the steam distillate, are treated with 6 drops of 1% glucose solution and concentrated sulfuric acid poured as a layer underneath. Positive reaction is a violet ring at the interphase. In weak concentration this reaction may show only after 10-12 hours. If the layering is not carefully done, strong heating may occur and caramelization simulate a positive reaction. The third test is an iodine-potassium iodide reaction: A few drops of KOH are added to 3 ml of the steam distillate, the mixture gently warmed, and concentrated iodine-potassium iodide solution added dropwise. A rose red color, or in higher concentrations a red precipitate, represent a positive reaction.

The sensitivity of these reactions was first checked with thymol solutions of known concentrations, as shown in table 1. Only part of the tested concentrations are shown.

b) Quantitative determination of thymol: According to Gordonoff and Janett, the colorimetric method for the determination of thymol by the diazo reaction as described by Pucher and Burd is not accurate enough for small amounts of material. We cannot agree with that opinion. The determinations, according to Pucher and Burd, are made by pipetting 3 ml of a 0.6% sulfanilic acid and 0.5 ml 2% HCl into test tubes, then adding 0.5 ml of a 1.5% sodium nitrite solution and after exactly 5 minutes, 1.5 ml test solution. Pucher and Burd warn never to add more than 0.5 mg thymol. In our determinations, the amount of thymol was always below 0.15 mg. After waiting one minute, the solution is made alkaline by the addition of 2 ml 3% NaOH. The color is compared to a standard thymol solution. We used standard thymol solutions of 1:10,000 and 1:100,000 in an Autenrieth colorimeter. The standard curves are not linear. Their course is shown in figures 1 and 2. It should be attempted to obtain readings in the steep parts of the curves. If necessary, a standard solution containing 1:50,000 will have to be used. The standard solutions should not be kept longer than 2 hours. We will return to these curves later.

According to Pucher and Burd, the thymol for the standard solutions should first be dissolved in a small amount of alcohol, then diluted with water. We did not obtain an acceptable standard curve by this method, apparently the solution is not homogeneous. We had not better luck when the used a small amount of ether for the preliminary solution. We recommend to accept the fact of the slower solubility in water, possibly to warm carefully on a water bath (30-35°C).

Here we like to mention an incidental observation which might be of interest in the evaluation of colorimetric methods. The color readings were always taken by two operators using separate standard curves. These two standard curves were always well parallel to each other. Due to the hyperbolic shape of the curves, the difference between the two readings was larger in the steep slope than in the shallower areas. Curves 1 and 2, the standard curves for thymol and carvacrol, show the difference between the readings of the two operators. The same relations exist for eugenol (curve 3) and guaiacol (curves 4 and 5). In the testing of the steam distillates from organs, plant extracts, etc., the values obtained from the readings of the two persons always agreed.

In addition to the Pucher and Burd method, thymol and carvacrol, singly and combined, were determined by the Vollmer procedure, where the Baeyer method for the detection of thymol and carvacrol as nitroso compounds has been adapted to a gravimetric determination. Here, the material is first steam distilled from an acid medium, the distillate shaken with ether, the ether evaporated, and the small aqueous residue made alkaline with NaOH. Then sodium nitrite solution is added and the liquid acidified. After standing in the refrigerator for 24 hours, the brownish precipitate is separated by filtration, then dissolved in ammonia and the nitroso compound reprecipitated with strong acetic acid. After standing in the refrigerator for 12 hours, it is filtered through a tared filter paper, dried at 40°C, and weighed. in this method which is meant for thymol samples of more than 6 mg, the error for low amounts is 20-25%, for high amounts, 15-20%. These values will therefore be somewhat lower than those from the colorimetric method.

The colorimetric method is preferable for samples containing only thymol, carvacrol, or a mixture of these two compounds. The gravimetric approach is suited to such cases where the presence of other substances which give a positive diazo reaction is suspected.

2. Determination of Carvacrol.

Carvacrol, being an isomer of thymol, also gives a positive diazo test and had to be determined together with it. The standard curves for carvacrol turned out to be identical to those of thymol (curves 1 and 2).

3. Determination of Eugenol.

Eugenol gives a red color with sulfuric acid. The dilution of the sulfuric acid interferes with this rather sensitive reaction when the sample is present in aqueous solution. When such samples are shaken with ether and the ether evaporated, there will be losses of eugenol. The chemical nature of eugenol made us expect that it would give a positive diazo reaction. This was indeed the case. Eugenol solutions down to 1:100,000 give a recognizable reaction.

concentrations to 1:85,000 can be determined colorimetrically. For optimum color development it is advisable to mix 3 ml 0.6% sulfanilic acid with 0.5 ml 2% HCl and to add 0.5 ml 1.5% sodium nitrite solution, wait 1 minute, add 3 ml test solution, wait again 1 minute, make alkaline with 1 ml 20% KOH and take a color reading after 10 minutes. Exact timing is imperative in the eugenol determination. The standard solution has to be used within 40 minutes. Curve 3 shows the calibration curve for eugenol.

4. Determination of Guaiacol.

Guaiacol can be determined by the diazo reaction just like thymol. Curves 4 and 5 which are included because of the earlier mentioned differences between the work for the two operators show the calibration curves for guaniacol.

5. Steam Distillation.

Finally, the steam distillation and the related control tests should be mentioned. We found, contrary to the statements of Rosenthaler, that thymol and carvacrol could not be steam distilled from alkaline medium with the type of material we were handling. But after distillation from weakly acid solution, we could account for all the thymol and carvacrol present. When larger amounts of thymol or carvacrol are mixed with ground up muscle material, practically all of it will be found in the steam distillate. There will be losses with smaller amounts. For instance, of 20 mg thymol mixed with 29 g muscle matter, 16 mg were found, of 20 mg added to 30 g liver pulp, 15 mg. We always distill organ material from a strong HCl solution.

In quantitative determinations, the distillations was kept up the disappearance of a positive reaction. Small samples were removed at intervals for this purpose. We found that the main fraction of the compound came over with the first milliliters of distillate. If large amounts are present, the first fraction may be turbid or contain oily droplets. These disappear as the amount of water increases. Where only qualitative determinations were needed, we would test the first 20 ml separately and would get a strongly positive reaction while a second sample would only give a very weak reaction. Eugenol may also be distilled from an alkaline solution, but the distillation from an acid medium is considerably faster.

TESTS.

1. Tests on Normal Animals

With reactions as delicate as those used by us, it was self-evident that a large number of normal animals had first to be tested. All animals received an unvarying diet of oats and potatoes. We examined 6 rats and 6 rabbits. Those tests with small doses in which steam distillation did not lead to positive reactions might be considered additional controls. We distilled from all animals lungs, liver, musculature, kidneys, stomach with content, entrails with content, and feces and urine of the rabbits only. In the case of the rats, all reactions after distillation from strong hydrochloric acid were negative. This refers to all three tests, the iodine-potassium iodide, carbohydrate, and diazo reaction.

From rabbits we never got a positive diazo reaction except from the content of stomach and entrails. After steam distilling these materials from strong HCl solution, a fugitive red color developed which changed to yellow, turned burgundy red on further standing, and finally faded. No reaction was observed after distillation from weak sulfuric acid solution. But as we used HCl only to recover chemically bound thymol completely, and as the thymol in stomach and entrails was known to be free, not yet resorbed, we could restrict ourselves in further tests to the distillation from weak sulfuric acid. Later tests will show that major amounts of thymol are not excreted through the entrails. The preliminary tests can be summarized by saying that in our method no foreign substances are included which might also give a positive diazo reaction.

2. Tests on the Fate of Thymol in the Organism.

a) Rat Tests. The amount of thymol that steam distilled from rat organs was generally too small for quantitative determination. We therefore ran only qualitative tests which, however were always compared to reactions with a series of known thymol concentrations. As mentioned earlier, the first 20 ml of distillate were tested. The first sample was taken after 8 ml had come over. Altogether, 8 rats were examined in this series. The tests are shown in table 2. Initially, we fed the rats high doses, but 500 mg thymol per 150 g body weight turned out to be lethal. After such doses death occurred 2-24 hours later with uncharacteristic symptoms. The eight tested animals received, as indicated in the table, 10 to 1000 mg thymol by urether catheter into the stomach and were killed after different lengths of time. Thymol, as well as all other substances, was dissolved in a small amount of sesame oil. Table 2 shows that the smallest dose after which thymol would be found in the lung was 20 mg. By running the test as described by Gordonoff, namely by adding the NaOH to the diazo reagent, we got a reaction which had to be termed dubious at best. The largest amounts were found in stomach and entrails.

b) Rabbit Tests: Initially, two rabbits were tested by qualitative reactions. These tests are charted in table 3. The first rabbit in the table had fed abundantly up to the time of the thymol administration. The second had been without food for 24 hours. Since differences might be due not only to different times of death but also to different amounts of feed, all animals in subsequent tests were kept without food for 24 hours prior to the test and did not receive any food during the test. This table shows that considerable amounts of thymol remained still in stomach and intestines, but because of the following quantitative tests on the distribution and excretion of thymol there will be no discussion here of the qualitative determinations.

Organs and excretions of six rabbits were tested by the quantiative method. These tests are shown in table 4. The table indicates the amounts of thymol found and also the calculated percentages based on total administered thymol. In the case of liver, lung, kidney, stomach with content, and entrails with content, the whole organ was distilled after careful preparation. The blood obtained from the bleeding of the carotids was also tested. As the thymol yield was low, we neglected to calculate it as part of the total blood content. For mulculature, we tested 30 g of thigh muscle material. The muscles also contained little thymol. By basing the calculation on a muscle content of 25% of total body weight, the amount of thymol there would represent approximately 10 to 25 times the amount given in the table. The value of 25% is based on some determinations where the complete musculature of an animal was separated and weighed. In evaluating this figure it should be remembered that even after several days of fasting there will still be a considerable amount of material in the rabbit's stomach and entrails. Feces and urine of two animals could not be separated because of their severe diarrhea. The urine was distilled from weak sulfuric acid in one case, from strong hyrochloric acid in another, in order to decompose any thymol that might be present in the form of an ester. As the table shows, the latter values are appreciably higher than the former. To be sure that this was really due to thymol, another sample was treated with concentrated hydrochloric acid, distilled, the distillate shaken with ether, the ether evaporated, and the thymol determined by Vollmer's method. The results from the colorimetric and the gravimetric methods was in good agreement. They were for one animal 607 mg by the colorimetric, 525 mg by the gravimetric method (based on total urine), where the gravimetric method is expected to be 15 - 20% in error.

Table 4 shows clearly that the lungs do not contain any appreciable amounts of thymol. Blood, liver, muscles, and kidney also contained little thymol. Larger amounts of thymol are found in liver and kidney only when the animal had been killed soon after the administration. By contrast we found 1.3-22% of the administered thymol in the stomach, 0.4-35% in the entrails, 30-60% in the urine. Resorbed thymol is very quickly excreted, excretion with the feces being of lesser importance. We also considered the frequently postulated excretion of thymol by way of the respiratory tract, about which we report in another part of this paper. Our animals exhibited the typical toxic symptoms described by Ellinger in Heffter's Handbook of Experimental Pharmacology, Vo. 1, page 929. The bronchopneumonias or inflammations of the respiratory tract, however, were never observed by us. One case of hemorrhagic urine indicated kidney damage.

3. Tests of the Fate of Carvacrol in the Organism.

As carvacrol is also a component of thyme, it too was considered in our studies. Since the conditions are similar to those of thymol, we limited ourselves to the examination of two rats, two rabbits and the carvacrol determination in the urine of a third rabbit. Table 5 shows the results of the carvacrol tests. Here too we find after 10-22 hours 8-16% still in the stomach, 22-32% in the entrails, while 26-40% have already been excreted with the urine. The carvacrol in the urine was determined by Vollmer's method. The results of the colorimetric and the gravimetric determination differed by 25.1% (391.4 and 293.3%, resp.). But the organs contained only little carvacrol. The rat tests at the head of the table show the same results. Carvacrol was administered in the same way as thymol by stomach tube and mixed with sesame oil to ameliorate its irritating properties. Carvacrol caused severe diarrhea. The animals developed paresis of the front extremities and shocklike effects immediately after administration. After lethal doses, death occurred with fast deterioration.

4. Tests with Extracts of Thymus Serpyllum.

Based on the tests described up to this point, a substantial buildup of thymol or carvacrol in the organs and especially the lungs must be considered impossible. Thymus serpyllum contains, according to the literature, up to 0.6% essential oil, 1% of which is thymol plus carvacrol. First, we determined the thymol and carvacrol content of the extracts to be used in the animal tests. The values obtained by the colorimetric and gravimetric methods are shown in table 6. We removed the alcohol from the extracts by careful warming in a vacuum, as we were going to administer large amounts to the animals and naturally had to avoid the possibility of alcohol poisoning. The loss of thymol and carvacrol in this procedure is shown in the table.

The results of the animal tests are given in table 7. Rats were given 5-7 g, rabbits, 50-100 g. Table 7 indicates to how much thymol plus carvacrol the doses corresponded. It is shown incontrovertably that the lungs of all animals contained no detectable amounts of thymol and carvacrol. There are traces of it in liver, kidney, and musculature. Larger amounts were found in stomach, entrails, and urine. The behavior of the animals after administration of the extract was normal.

5. Remarks to Paragraphs 2,3 and 4.

The tests as reported up to here indicate that after administration of thymol, carvacrol, or extracts of thymus serpyllum the steam distillate from the lungs of the test animals did not give a strong diazo reaction, and that the quantitative results for thymol were very low. Since we worked always with the complete organs, the objection might be made that the weight of the lungs is only a small fraction of that of the liver so that the figures are not comparable. This objection is invalidated by the figures in table 8. By basing the calculated amount of thymol on 100 g of organ matter, we can show that, a short time after administration of thymol or carvacrol, the liver contains far more of these substances than the lung, and that after a longer time the two figures are approximately the same The corresponding values for the kidney are higher than those for lung or liver. This further points to the importance of the kidney as the organ of excretion. This approach does not indicate either that there is any buildup of thymol or carvacrol in the lung

6. Test on the Excretion of Thymol and Carvacrol

by the Lung.

Husemann observed inflammation of the respiratory tract after administration of large amounts of thymol and drew the conclusion that there was excretion by the lung. Apart from some more thorougly studied substances like alcohol and acetone, there is scant knowledge about the function of the lung as organ of excretion. Considering that many substances that are supposed to be excreted by the lungs have a characteristic odor even at great dilution, and that they have been taken by mouth, the conclusion that the odor of the exhaled air proves excretion cannot be considered valid. We therefore attempted here, as well as later with eugenol and guiacol, to resolve the question of possible excretion by the lung in an objective way.

At the start, rats were placed in a glass vessel through which an air stream was drawn which then passed through three ice cooled wash bottles with alcohol. After tests that ran for hours, using rats that had been given 500 mg thymol or carvacrol, we found at best traces in the first wash bottle, but most of the time nothing. Since the traces (reactions corresponding to approximately 1:1,000,000) might also be due to excreta, this possibility was eliminated in the rabbit tests.

The rabbits were placed inside a box, their heads sticking out through a round window that matched the circumference of their necks. The head was placed inside a metal box corresponding to their head size and this sealed against the neck with waterproof muslin. At the bottom of the metal box was an air inlet tube, on the opposite side another tube by which air could be removed by suction. The air stream was drawn by water aspirator through several ice cooled washbottles. The apparatus was checked by using it on a rabbit to which 2000 mg acetone had been administered by subcutaneous injection. After 22 hours, 590 mg acetone were determined in the wash bottles by the customary iodometric titration. A subsequent shorter run indicated that the excretion of the acetone was not yet complete. After thus proving the serviceability of the apparatus, we ran tests on rabbits that had received 1500 - 5000 mg thymol or carvacrol. With rabbits that had received 1500 mg thymol, only traces were found after 10 hours, with a rabbit that had been fed 5000 mg thymol, 0.3 mg were found after 22 hours. For carvacrol, after a test with 1500 mg and 8 hours, 0.4 mg were found, in another with 5000 mg and 12 hours, also 0.4 mg. This animal died after 12½ hours. Needless to say that after each test the metal boxes and short tubing were carefully washed with alcohol. These tests prove that there is no major excretion by the lungs. An explanation for the small amounts that were found may be that after administration small amounts of the substances adhered to the stomach tube and were deposited in the mouth cavity after its withdrawl. The lack of builup in the lungs also speaks against excretion through the lungs, in contrast to the kidneys.

7. Tests on the Excretion and Distribution of Eugenol.

Since the tests on thymol and carvacrol had shown a similarity of these substances in the behavior in the organism, it could be assumed that other higher phenols of similar structure would act in an analogous way. As additional representative of this group of compounds we chose eugenol, the main constituent of clove oil, chemically the monomethyl ether of allylpyrocatechol. As table 9 attests, the results were indeed quite similar. Here too, amounts in excess of lung of eugenol appeared in the organs only a short time after administration of large doses. The exhaled air was also tested as described above on two rabbits ans several rats. Even after fairly large doses (1500-5000 mg), no eugenol could be detected in the receiving vessels from either type of animal. Eugenol had a definitive narcotic effect. The corneal reflex disappeared ½ hour after administration of 1500-5000 mg to rabbits. After small doses, the narcosis would last for hours, but only large doses lead to death.

8. Tests on the Excretion and Distribution of Guaiacol.

A number of publications exist on guaiacol and its excretion. Fonces and Diacon determined excretion of 66% in the urine. Eschle's values are similar. Hensel estimates the amount to be 86%. Because of the odor of the exhaled air, here too excretion by the lungs has been postulated, but this assumption was rejected by Marfori and Bufalini.

Neither could we ever get a positive reaction from the content of the receiving vessels of our apparatus, even after 24 hour tests with large doses of guaiacol. The distribution and excretion in urine and feces was checked on three rabbits. The test results are shown in table 10. We receovered 85% of the administered guaiacol in the urine. Here too the organs contain major amounts only in animals that had been killed soon after administration. Here as in the previouslyexamined substances, the excretion of the resorbed guaiacol by the kidney occurs very fast, while excretion with the feces is insignificant (0.03 and 0.04% of the administered dose). Only minor amounts were detected in the blood.

9. Solids Determinations on Lung and Liver of Normal Guinea Pigs and such Treated with Thymol, Carvacrol, Eugenol, Guaiacol, and Potassium Sulfoguaiacolate.

As mentioned in the beginning, Vollmer examined the effect of different expectorants on the solids content of lung and liver of guinea pigs. He found a lowering of the solids content by saponines, extracts of Castanea vesca, and potassium sulfoguaiacolate. Of his other results should be mentioned that extracts of thymus serpyllus did not change the solids content of the lung.

We tested the effect of larger doses of thymol, carvacrol, eugenol, and guaiacol by the same method and included, for comparison, also potassium sulfoguaiacolate. It is necessary in these tests to work with fully grown animals, since, as Vollmer showed, the dehydration of the organism which occurs in the course of an animal's life is clearly expressed in the different values for the solids content of guinea pigs of different sizes. Other tests by Vollmer also show the importance of the greatest homogeneity of the animal material as to size of specimens in all biological test series. We used animals of 400-600 g body weight, as the water content of guinea pigs weighing more than 350 g is rather constant, or anyway not lower than certain given values. The drying was done for 4½ hours in a drying oven at 104-108°C. The substances were given to the animals by mouth by means of a urether catheter, the animals killed 120 minutes later with illuminating gas, the thorax immediately opened, the content excised, heart, oesophagus and blood vessels, also the trachea up to the bifurcation removed, and the lungs placed in a glass weighing dish after superficial blotting with filter paper. A piece of liver weighing 2-3 g was used as sample. In Vollmer's test series, a total of 37 control animals were killed which averaged 20.8% solids in the lungs, 28.7% in the liver. The ratio of lung to liver averaged 0.72. Our results obtained from 10 normal control animals agreed well with these older values, as table 11 indicates.

Table 12 gives the values for the animals treated with the above mentioned compounds Each was given a 100 mg dose in 2 ml sesame oil. Potassium sulfoguaiacolate was tested along with the rest because the guaiacol animals showed, against expectations, no deviation from the norm. This result corresponds to earlier tests where potassium sulfoguaiacolate in aqueous solution caused only slight water enrichment in the lung. The results in table 12 show another effect that had not been observed earlier with the smaller doses: The solids content of the liver of animals fed thymol and potassium sulfoguaiacolate is appreciably, those fed carvacrol and eugenol moderately, lowered, while the guaiacol animals show no change whatsoever. Where the solids content of the liver was lowered, the gall bladder was heavily filled, an observation that had also been made in the previously mentioned test series with thymol, carvacrol, and eugenol. It is likely that the water increase in the liver of these animals is connected with the well known excretion of the administered substances by the gall bladder. Striking is the difference between the animals that received guaiacol and those that got potassium sulfoguaiacolate, and between thymol and carvacrol animals. The lung/liver ratio which appeared so characteristic in the earlier series loses its value by the simultaneous effect on the liver.

Mention should here be made of the fact that the lowering of the solids content of the lung has no bearing on the efficacy of the substance as expectorant. In determining the dried solids content, only a partial effect of the mechanism is dealt with, and the negative results with two effective expectorants, potassium iodide and ammonium chloride, show sufficiently that there are other mechanisms at work. At any rate, the determination of the solids content is able to give us certain hints. It is remarkable in this series that two frequently used expectorants, thymol and potassium sulfoguaiacolate, cause an increase of water in the lung.

SUMMARY

1) The diazo reaction is well suited for the colorimetric determination of thymol, carvacrol, eugenol, and guaiacol in the steam distillate from organs

and excretions of rats and rabbits. Details of the determination are given.

2) 25-95% of thymol, carvacrol, eugenol, and guaiacol given by mouth are resorbed within 24 hours. The resorption is quicly followed by excretion in the urine. Excretion in the feces plays only a very minor role. Data are given for the excreted, not yet resorbed fraction, as well as for the amount of the substances remaining in the organs at different length of time after administration.

3) Only during the first hours after administration are significant amounts of any of the substances found in blood, lungs, kidney, and liver.

4) An accumulation in the lung was not observed with thymol, carvacrol, eugenol, or guaiacol.

5) Only traces of the tested substances were excreted by way of the respiratory tract even after very high doses.

6) The determination of the solids content of lung and liver of guinea pigs showed accumulation of water in the lung after administration of thymol and potassium sulfoguaiacolate, while the other compounds did not influence the water content of the lung. However, thymol, carvacrol, eugenol, and potassium sulfoguaiacolate lower the solids content of the liver. This accumulation of water is very likely due to the excretion of the test compounds by the gall bladder.

TABLE I

The Sensitivity of Different Thymol Reactions.

Concentration	I_2-KI Reaction	Carbohydrate Reaction	Diazo Reaction
1: 5,000	++	++	+++
1: 15,000	+(+)	++	+++
1: 35,000	?	++	+++
1: 50,000	--	+(+)	+++
1: 75,000	--	+	++(+)
1:100,000	--	+	++
1:200,000	--	--	++
1:500,000	--	--	+
1:800,000	--	--	+

The designations were chosen as follows:

Iodine-Potassium iodide reaction:
- ++ Immediate rose red precipitate
- +(+) Precipitate after some time
- + Rose red color

Carbohydrate reaction:
- ++ Immediate ring formation
- + Ring forms only after longer standing

Diazo reaction:
- +++ Strong reaction, reddish yellow color
- ++ Reaction predominantly yellow with hint of red
- + Yellow color

TABLE II

Thymol Reaction in Steam Distillates from Organs of Rats that had been Fed Different Amounts of Thymol.

Rat. No.	Hours after death	Thymol Dose in mg	Lung J	Lung K	Lung D	Liver J	Liver K	Liver D	Stomach J	Stomach K	Stomach D	Entrails J	Entrails K	Entrails D	Other Organs	Other Organs J	Other Organs K	Other Organs D
1	3	1 000	-	-	+	+	+(+)	++	++	++	+++	++	++	+++	Feces	-	+	++
2	1 3/4	500	-	+	++	-	+	++	++	++	+++	++	++	+++				
3	2	500	-	+	++	-	+	++	++	++	+++	++	++	+++	Kidney	-	+	+
4	21	100	-	-	+	-	-	-	+	++	+++	-	-	++				
5	15	20	-	-	+	-	-	+	-	+	+++	-	+	++	Musculature	-	-	-
6	24	10	-	-	-	-	-	-	-	-	++	-	-	++	Musculature	-	-	-
7	18	10	-	-	-	-	-	-	-	-	-	-	-	+	Feces	-	-	(+)
8	18	10	-	-	-	-	-	-	-	-	+	-	-	+				

J = Iodine-potassium iodide test

K = Carbohydrate test

D = Diazo test

Strength of reaction indicated as in table 1.

TABLE III

Thymol Reaction in the Steam Distillates from the Organs of two Rabbits Treated with Thymol.

Rabbit No.	Lung			Liver			Blood			Muscle			Stomach			Entrails			Kidney and Urine		
	J	K	D	J	K	D	J	K	D	J	K	D	J	K	D	J	K	D	J	K	D
1*	-	-	(+)	-	-	-	-	-	-	-	-	(+)	+	++	+++	+	++	+++	-	-	+
2**	-	+	+	-	+	+	-	-	-	-	-	+	+	++	+++	+	++	+++	-	++	+

Designations as in Table I

* 2.2 kg weight, normal copious food. After dissection stomach very rull.

Death after 8 hours. Thymol dose: 1000 mg.

** 1.84 kg weight. No food 24 hours before administration of thymol and afterwards.

Death after 20 hours. Thymol dose: 1000 mg.

TABLE IV.

Distribution and Excretion of Thymol Administered to Rabbit by Mouth.

Rabbit No.		1	2	3	4	5	6
Death after hours						4 hours after last dose	Death after 18 hours
Thymol dose in mg						1000 mg each on 3 succeeding days	200
Lung . . .	mg	0.06	2.7	0.06	traces	2.3	<0.1
	%	0.006	0.09	0.006	-	0.08	-
Liver . .	mg	0.06	8.4	<0.04	traces	24.5	<0.1
	%	0.006	0.28	-	-	0.82	-
Kidney . .	mg	0.1	6.3	0.1	0.6	11.5	0.6
	%	0.01	0.21	0.01	0.06	0.38	0.3
Stomach .	mg	22.0		72.3	13.2	465.8	44.0
	%	2.2		7.23	1.32	15.53	22.0
Entrails .	mg	4.3		11.1	17.0	1043.8	33.3
	%	0.43		1.11	1.7	34.79	16.6
Feces . .	mg			1.1	traces		0.0
	%			0.11	--		0.0
Urine, dist. fr HCl	mg			607.2	509.6	1019.3	110.9
	%			60.72	50.96	33.97	55.5
Urine dist. fr. H_2SO_4	mg	300.0		205.0	197.6		26.7
	%	30.0		20.5	19.76		13.4
Muscle . .	mg	0.06	<0.48	0.06	traces	3.4	0.0
	%	0.006	0.0016	0.006	--	0.11	0.0
Blood . .	mg	0.06		0.06	0.3		<0.1
	%	0.006		0.006	0.03		-
Total collected	mg	326.64	17.88	691.98	540.7	2570.6	188.8
	%	32.66	0.60	69.20	54.07	85.68	94.4

Amount of recovered thymol given in milligrams and as percentage of the administered thymol.

TABLE V.

Tests on the Distribution and Excretion of Carvacrol.

Rat		1	2	Rabbit	1	2	3
Death after hours		2	20		22	10	18
Carvacrol dose in mg		500	50		1500	5000	1500
Lung	J	-	-	mg	8.4	20.9	
	K	-	-	%	0.56	0.42	
	D	+(+)	+				
Liver	J	-	-	mg	17.6	41.0	
	K	+	-	%	1.17	0.82	
	D	++	+				
Kidney	J	-	-	mg	9.7	30.9	
	K	-	-	%	0.65	0.62	
	D	++	+				
Stomach	J	-	-	mg	130.4	834.6	
	K	++	++	%	8.69	16.69	
	D	+++	+++				
Entrails	J	-	-	mg	333.4	1638.1	
	K	++	+	%	22.22	32.76	
	D	+++	++				
Feces	J			mg	19.1		
	K			%	1.27		
	D						
Urine	J	-	-	mg	391.4	1560.0	595.5
	K	++	+	%	26.09	31.2	39.71
	D	+++	+++				
Muscle	J	-	-	mg	1.8	14.9	
	K	+	-	%	0.12	0.30	
	D	++	-				
Blood	J			mg	24.5		
	K			%	1.63		
	D						
Total collected				mg	963.3	4140.4	595.7
				%	62.4	82.8	39.7

Amount of carvacrol receovered given in milligrams and as percentage of the administered material. Results of qualitative tests on rats designated as in table 1.

TABLE 6.

Determination of Thymol and Carvacrol Content of

the Extracts of Thyme Used in the Tests.

No.	Preparation	mg thymol in 100 g extract		Remarks
		Pucher-Burd	Vollmer method	
I	Thymus serpyllus extracted with 22% alcohol and 3.6% glycerol	33.4	32.2	Averages of 4 determinations each
II	Extract I after removal of alcohol and replacement with water	24.0	--	Average of 2 determinations
III	Alcoholfree extract of thymus serpyllus	5.4	4	Averages of 3 determinations each

TABLE 7.

Thymol Plus Carvacrol Found in Organs and Excretions of Rabbits and Rats after Administration of Extracts of Thyme.

	Rat No. 1 and 2	Rat No. 3 and 4	Rabbit No. 1	Rabbit No. 2	Rabbit No. 3
Death after hours	5	7	22	5	22
Extract dose in g	5 each, extract I	7 each extract II	100 extract III	50 extract III	2x50 Extract II within 9 hours
Corresponding to mg thymol and carvacrol	3.4	3.36	5.4	2.7	24
Lung	-	-	-	-	-
Liver	-	-	traces	traces	-
Kidney		-	-	traces	-
Stomach	++	+	-	0.3	4.6
Entrails			0.5	0.2	-
Feces	-	+	traces	-	0.5
Urine	-		4.5	2.0	17.8
Muscle	-	-	-	-	-
Blood	-	-	-	-	-

Recovered amount of thymol given in milligrams of administered thymol.

TABLE 8.

Calculation of the Amounts of Thymol or Carvacrol Recovered in the Steam Distillates from Rabbit Tests as Milligrams Thymol in 100 g Organ.

	Rabbit 2, Table 4	Rabbit 5 Table 4	Rabbit 1 Table 5	Rabbit 2 Table 5
Death after hours	40	4 hours after last dose	22	10
Thymol or Carvacrol in mg	3000	1000 each on 3 succeeding days	1500	5000
Lung	17.9	16.4	33.5	58.2
Liver	17.4	44.9	34.4	71.7
Kidney	40.0	88.5	99.4	140.5

TABLE 9.

Determination of the Distribution and Excretion of Eugenol.

	Rat No. 1 and 2		Rabbit No. 1	Rabbit No. 2	Rabbit No. 3	Rabbit No. 4
Death after hours	22		20	5	15	4
Eugenol dose in mg	500 each		2,500	5 00	1,500	1,500
Lung	(+)	mg	<1.0	14.7	-	-
		%		0.29	-	-
Liver	+	mg	<1.0	66.7	trace	-
		%		1.33	-	-
Kidney	+	mg	<1.5	42.3	<0.8	8.2
		%		0.85		0.59
Stomach	+++	mg	1102.5	3467.0	287.5	567.9
		%	44.1	69.34	19.17	37.81
Entrails	+++	mg	<1.5	418.7	13.2	139.5
		%		8.37	0.88	9.30
Feces	+++	mg	+	-	44.4	-
		%		-	2.96	-
Urine from HCl		mg	+++	20.3	643.3	632.8
		%		0.41	42.89	42.19
Urine from H_2SO_4		mg	+++		333.3	487.8
		%			22.22	32.52
Muscle	(+)	mg	<1.0	14.3	-	-
		%		0.29	-	-
Blood		mg	<1.0	41.0	trace	<0.8
		%		0.82	-	-
Total collected		mg	1102.5	4085.6	988.4	1348.5
		%	44.1	81.7	65.9	89.9

Amount of recovered eugenol given in milligrams and as percentage of administered eugenol.

TABLE 10.

Determination of Distribution and Exretion of Guaiacol.

Rabbit		1	2	3
Death after hours		2½	12	20
Guaiacol Dose in mg		1,500	1,500	1,500
Lung. . .	mg	8.9	trace	trace
	%	0.59	-	-
Liver . .	mg	90.4	0.5	trace
	%	6.03	0.03	-
Kidney .	mg	41.1	0.6	0.5
	%	2.74	0.04	0.03
Stomach .	mg	973.7	71.4	83.8
	%	64.91	4.76	5.59
Entrails .	mg	178.5	1.4	1.7
	%	11.9	0.09	0.11
Feces	mg	-	0.6	0.4
	%	-	0.04	0.03
Urine from HCl	mg	71.2	1276.6	1204.2
	%	4.75	85.1	80.28
Urine from H_2SO_4	mg	52.9	1052.6	444.0
	%	3.53	70.17	29.6
Muscle	mg	12.6	trace	trace
	%	0.84	-	-
Blood	mg		trace	trace
	%		-	-
Total Recover	mg	1376.4	1351.1	1290.6
	%	91.76	90.07	86.04

Amount of recovered guaiacol given as milligrams and as percentage of administered guaiacol.

TABLE 11.

Dry Solids Content of Lung and Liver of Ten Untreated Guinea Pigs

Expressed as Percentage and as Lung: Liver Ratio.

Guinea Pig No.	Lung	Liver	Lung :Liver
1	20.8	29.7	0.70
2	20.7	28.3	0.73
3	21.5	29.3	0.73
4	20.0	27.3	0.73
5	21.7	28.7	0.76
6	23.2	27.7	0.84
7	20.2	27.5	0.73
8	21.1	27.8	0.76
9	21.7	29.8	0.73
10	20.9	28.7	0.73
Average	21.2	28.5	0.74

TABLE 12.

Dry Solids of Content of Lung and Liver of Groups of Four Ginea Pigs Each Treated with Thymol, Carvacrol, Eugenol, Guaiacol, and Potassium Sulfoguaiacolate, Expressed as Percentage and Lung:Liver Ratio.

Treated with	Animal No.	Lung	Liver	Lung: Liver	Average Lung	Average Liver	Average Ratio
Thymol	1	20.1	24.7	0.81	17.5	24.8	0.71
	2	14.8	26.5	0.56			
	3	15.4	24.4	0.63			
	4	19.8	23.4	0.85			
Carvacrol	1	20.9	27.9	0.75	21.0	26.1	0.81
Carvacrol	2	21.7	25.7	0.84			
	3	21.8	24.8	0.88			
	4	19.8	26.0	0.76			
Eugenol	1	21.0	27.9	0.75	21.3	26.8	0.73
	2	21.1	29.9	0.71			
	3	22.0	31.3	0.70			
	4	21.1	27.8	0.76			
Guaiacol	1	20.1	26.2	0.76	20.0	27.8	0.71
	2	20.9	28.7	0.71			
	3	18.8	27.6	0.68			
	4	20.3	28.9	0.70			
Potassium sulfoguai-acolate	1	19.8	25.2	0.70	19.1	25.4	0.75
	2	19.2	26.4	0.73			
	3	17.4	24.7	0.70			
	4	20.0	25.4	0.79			

The animals received 100 g each of the substance in 2 ml sesame oil and were killed 120 minutes later.

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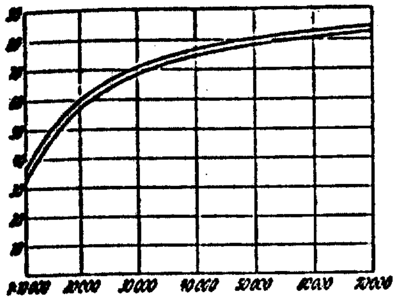

Fig 1

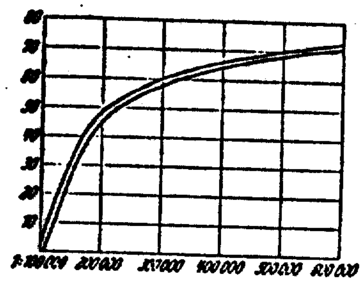

Fig 2

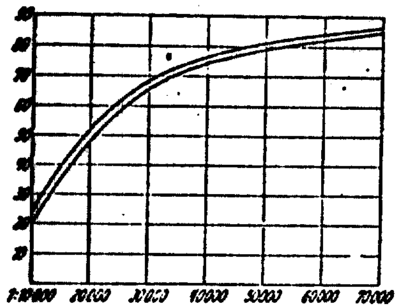

Fig 3

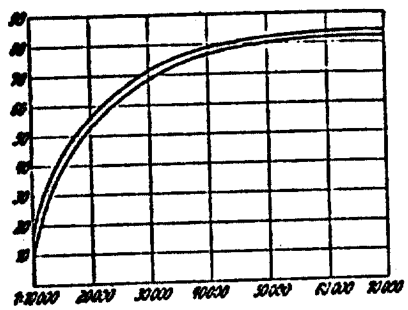

Fig 4

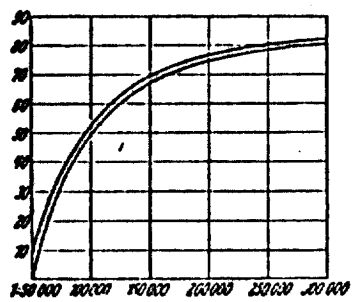

Fig 5

TABLE I.
Skin Lesions and Riboflavin Content of the Liver Associated with Various Amounts of Riboflavin (R) and 2-acetylaminofluorene (AAF) in the Diet.

Group	R, γ/30 g diet	AAF %	Casein, %	Skin lesions	Liver riboflavin, γ/g tissue
1	3	0	18	—	16.0 ± 1*
2	3	.03	18	++++	9.5 ± 1
3	0	0	18	++	12.7 ± 0.9
4	0	.03	18	+++	9.3 ± 2
5	30	.03	18	—	16.0 ± 1.5
6	50	.03	0	+	9.9 ± 1

* Standard error.

ficiency, keeping the liver riboflavin at normal levels. In the absence of protein in the diet, however, the increased riboflavin did not prevent the development of the deficiency.

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Toxicity of Eugenol: Determination of LD50 on Rats.* (17608)

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As a result of the demonstration that eugenol (4-allyl-2-methoxyphenol) is a very effective mucigogue and desquamatory agent in the stomach,(1) this substance has been used for the study of mucus secretion and gastric cytology in man, without gastric resection or gastroenterostomy.(2) Our technic involves administration of an aqueous emulsion of the eugenol by stomach tube, and its reaspiration after about 15 minutes. Prior to such clinical application, however, it was deemed necessary to estimate the limits of safe dosage for this substance. Such a study, conducted on dogs without any gastric operation,(3) revealed a maximum safe dosage of about 0.2 g/kg body weight, when an aqueous emulsion was administered in this same manner without reaspiration. A dose of 0.25 g/kg caused no fatalities, but induced vomiting in 2 of 7 experiments. There was no gross or microscopic evidence of any cumulative effect after repeated administration of this dose—10 times in the course of 3 weeks. At dosage levels around 0.5 g/kg, reactions to the irritant, including vomiting and ataxia, were manifest and in 2 of 6 experiments, the dog died within 24 hours. Hence, a more precise determination of the toxicity, carried out on a large number of animals, was necessary. Such a study on rats is presented herein.

Experimental Procedure. About 200 albino rats, each weighing approximately 200 g, were used in this investigation. The animals, kept in individual wire cages, were fasted for 24 hours before the eugenol was administered and for about 4 hours thereafter. A total of 91 animals were included in the final dosage-mortality analysis. The individual dosage groups were so constituted that each contained equal numbers of males and females. Eugenol (Merck, U.S.P.) was administered undiluted in a single dose, in a manner essentially the same as that reported by Shay(4)

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1. Hollander, F., and Lauber, F. U., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 34.

2. Hollander, F., Hess, M., and Sober, H. A., *J. Nat. Cancer Inst.*, 1947, v7, 365.

3. Lauber, F. U., and Hollander, F., *Gastroenterology*, in press.

4. Shay, H., and Gruenstein, M., *J. Lab. Clin. Med.*, 1946, v31, 1384.

for intra-gastric instillation in rats. If any resistance to passage of the catheter was encountered, the tube was removed immediately and passed a second time. Only 2 of some 200 rats were lost as a result of the intubation, and these 2 deaths occurred within 10 seconds after administration of the material. Since such accidental deaths were easily recognized, they were omitted from the results reported. All animals were observed closely throughout the day of administration of the eugenol, and were checked frequently for at least 10 days thereafter. All but 2 of the deaths occurred during the first 2 days. Those rats in which postmortem changes were considerable, because of death during the night, were discarded; all others were autopsied as soon after death as possible, and tissues from representative animals were taken for microscopic examination. Eugenol was administered in dosages based on body weight. In order to approximate the dosage range suitable for a dosage-mortality study, preliminary runs were made with several groups of 2 rats each. Based on these observations, more extensive runs were made in the range 1.5-2.2 ml/kg (1.6-2.3 g/kg) using 12 animals in each group. Whenever any doubt arose regarding the completeness of administration of the eugenol, the animal was immediately discarded from the group.

Results. Toxic manifestations. The first observable effect after the administration of eugenol was a weakness of the hind legs, which was seen in 100% of the animals. As a rule, this appeared in about 5 minutes, and by 15 minutes, there was complete paralysis of the lower extremities. Simultaneous with, or just after the onset of this hind leg effect, the lower jaw relaxed and remained in an open position for a variable period of time. This was observed in 70 (86%) of the animals. Sixty-four (70%) finally became prostrate and were unable to move, though eye reflexes were still present; 54 of these prostrate animals (60% of the total) went on to complete coma. In no case was an animal observed in which the front legs were affected before prostration or coma set in. Only 5 animals survived the coma; of the remaining 49, at least 10 were observed to arouse one or more times before death. During coma, breathing was irregular, changing from slow, deep, gasping excursions of the chest, to rapid, weak, abdominal respiration. The extremities became cyanotic and cold. Cessation of respiration usually occurred before heart failure. The time of death varied from 1 to 48 hours, 41% of all the deaths occurred within the first 6 hours, 33% in the next 18 hours, and 22% during the second day after administration of the eugenol. One animal died on the third day and one on the sixth. All survivors, whether they became comatose or not, were lethargic and manifested some degree of narcosis—some for as long as 3 days. On the second or third day, the animals usually sat hunched in a corner of the cage. After being prodded, they walked with hunched back and exaggerated high-step of the hind legs. Fifty per cent developed hematuria or urinary incontinence by the third day. By the fifth day, the survivors had all essentially recovered.

Pathology. In 38 (95%) of the 40 animals which were autopsied, eugenol odor was detected in either the stomach, the duodenum, or both. The blood vessels in the peritoneum and mesentery were engorged, as were those in the diaphragm, ribs, and genital organs. The peritoneal wall was glistening and diffusely inflamed, varying from pink to bright red, and fluid was present in the peritoneal cavity. There was diffuse congestion of the kidneys, and the medulla was often pink and poorly delineated. The liver also was usually congested and darker than normal. The glandular stomach contained mucus, clotted blood, and occasionally some areas of mucosal erosion; the fore-stomach appeared normal. Blood clots were often found in the lumen of the small intestine. The lungs were usually mottled and redder than normal. In many animals opened promptly after death, the auricles were still beating; in all instances, the ventricles were engorged and the vessels were injected. The general impression was that of a diffuse inflammation with congestion in the viscera.

The microscopic studies yielded evidence of pathologic change, especially following the use

TABLE I.
Dosage-Mortality Data for Eugenol.

Group No.	Eugenol dose, ml/kg	No. of rats per group	No. of deaths following administration (by days)					
			1*	2	3	4-10	Total	%
T-13	1.5	12	2 (2)	2	—	—	4	33
20	1.6	12	6 (3)	1	—	—	7	58
14	1.75	11	0 (0)	2	—	1†	3	27
16	1.75	11	1 (0)	1	1	—	3	27
17	1.9	12	3 (1)	1	—	—	4	33
15	2.0	11	7 (5)	1	—	—	8	73
22	2.1	11	8 (6)	1	—	—	9	82
21	2.2	11	9 (3)	2	—	—	11	100
	Total	91	36 (20)	11	1	1	49	
			74%	22%	2%	2%	54%‡	

* Values in parentheses give number of deaths in first 6 hrs.
† This death occurred on the sixth day.
‡ This represents the proportion of all deaths among all the animals treated, whereas the other percentages on this line give the proportions of all the deaths which occurred in the groups indicated.

of the highest dosages (2.1 and 2.2 ml/kg). Damage appeared to be greatest in the lungs, liver, kidney, stomach and duodenum, with relatively minor or no changes elsewhere. The lungs were emphysematous, with distended bronchi, and intense congestion was evident in the larger blood vessels as well as in the alveolar septa. In the liver, extensive congestion of the central veins and sinusoids was observed. At the higher dosages, the kidneys showed evidence of mild tubular dilatation, with the tubular epithelium swollen and distended with blood. The effects of eugenol on the nervous system in general were not studied histologically, but the possibility of their occurrence must be entertained in view of the gross observation of narcosis and malfunction of limbs and jaw following administration of the irritant. The wall of the forestomach was normal, showing no apparent reaction to the eugenol. The glandular stomach mucosa sometimes showed evidence of subacute inflammatory reactions with extensive leucocytic infiltration. In general, the columnar cells were distended with mucus, and desquamated masses of surface epithelium were noted in the lumen of the viscus. In the mucosa of animals given high dosages, areas of desquamation were distinctly visible. Parietal cells were well preserved, but the peptic cells were degenerate in appearance. Duodenitis was sometimes seen. There was evidence of excessive mucus production not only in the stomach but also in the duodenum and other areas of the small intestine.

LD50. The mortality data are summarized in Table I. Using the statistical technique described by Bliss(5,6) the LD50 for eugenol in albino rats under the conditions herein described was found to be 1.8 ml/kg (1.93 g).

Antagonistic agents. Several stimulants were tested for possible therapeutic action in animals poisoned with eugenol: Picrotoxin (1 mg/kg), coramine (5 mg/kg), strychnine (0.75 mg/kg), caffeine (10 mg/kg), and metrazol (7.5 mg/kg). Each of these was tested on a group of about 10 rats that had received eugenol in single doses of 2.0 ml/kg. The drugs were injected intraperitoneally when the animals were already in coma or, if no coma occurred, when they were in deep narcosis. In some animals, repeated injections of the same dosage were made. No statistically significant reduction in mortality was observed in any of these experiments.

Summary. The LD50 for pure eugenol, administered to rats by stomach tube, and without subsequent reaspiration, was estimated to be 1.8 ml (1.93 g/kg). Any use of this datum as a guide to safe dosage in man

5. Bliss, C. I., *Ann. Appl. Biol.*, 1935, v22, 134.
6. Bliss, C. I., *Quart. J. Pharm. Pharmacol.*, 1938, v11, 192.

however, may take cognizance of the fact that the conditions of the rodent studies are considerably more severe than any which might be encountered in clinical studies. In the first place, the rat experiments employed eugenol *per se*, whereas work with dog and man is done with aqueous emulsions of the irritant. Furthermore, the rat retains all of the eugenol administered, because of its inability to vomit, whereas dog and man can reject part of the irritant in this manner if the dose is excessive, and this affords an additional factor of safety.

The salient features of the toxic manifestations of eugenol in the rat were as follows: Paralysis occurred initially in the hind legs and the lower jaw. The forelimbs were unaffected unless general prostration or coma ensued. In those animals in which the acute symptoms subsided, the animals remained lethargic, showed signs of urinary incontinence and frequently hematuria, and gave evidence of impaired function of the hind legs for several days. Gross and microscopic observations of the tissues suggested that profound changes in fluid distribution had occurred in response to acute irritation of the gastrointestinal tract. The net impression of the effect of the eugenol, from the microscopic evidence, was essentially that of circulatory collapse with resultant congestion.

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RESPONSE OF GASTRIC MUCOUS BARRIER IN POUCH DOGS TO REPEATED TOPICAL APPLICATION OF EUGENOL[1, 2]

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THE major function of the gastric 'mucous barrier,' comprising the easily desquamated mucous epithelium as well as the layer of viscous mucus covering it, is to protect the underlying tissue against irritation. Such protection rests on the particular physico-chemical properties of the secretion (i.e. viscosity, cohesiveness, buffering capacity and enzyme adsorption), and also the rapidity with which the surface epithelium can be exfoliated and replaced (1, 2). Our previous studies of these phenomena were concerned with the response of the dog's mucosa to one or occasionally two successive applications of various mild irritants (3–5), including aqueous emulsions of eugenol at different concentrations. In all such experiments, except with mustard oil, the physico-chemical properties of mucus collected from gastric (Heidenhain) pouches varied extensively. Since it usually contained cellular material in addition to extra-cellular secretion (transparent and translucent specimens were cell-free), its opacity was ascribed to desquamated columnar cells (and leucocytes) as well as coagulated mucin. In general, it was inferred that this desquamation, like the secretory response itself, is a definitive, but not necessary, reaction to even very mild stimulation. Variations in physico-chemical characteristics were ascribed to differences in mucin content and also to admixture of serous transudate, exudate, blood, and possibly a mucoid secretion.

The present investigation is concerned with *repeated* stimulation of the gastric mucosa with eugenol in order to *1*) investigate the possibility of fatiguing or exhausting the mucous secretory apparatus, *2*) gauge the extent of damage produced by repeated insult to the mucous barrier, and *3*) determine the recovery of function after some lapse of time. Such information is expected to prove of value in interpreting clinical reports of a deficiency in gastric mucous secretion. Also, it may afford a new experimental approach to some of the problems of gastric disease—notably, gastritis, peptic ulcer, and carcinoma of the stomach.

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[1] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. P. H. S.

[2] Preliminary reports of this work were presented before the Third National Gastric Cancer Conference held in Chicago, December 5–6, 1946 (6) and before the American Physiological Society in 1947 (7).

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EXPERIMENTAL PROCEDURE

The reasons for selecting eugenol (4-allyl-2-methoxyphenol, Merck, U.S.P.) are detailed elsewhere (3). The stimulus was freshly prepared each day as a 5 per cent aqueous emulsion which remained stable throughout the experiment. The emulsion, containing Tergitol Penetrant-4[6] (1 drop/100 ml.), was made with a hand homogenizer.

Gastric corpus pouches of the Heidenhain type were prepared in 5 mongrel bitches of medium weight. One of these (*dog 169*) was used only for trial experiments with 2 per cent eugenol; all subsequent work employed 5 per cent emulsions. Experiments were started at least 2 months following surgery, and the animals were never used for any other purpose. The dog was deprived of food for 18 to 24 hours preceding the experiment, and was given 15 ml. of castor oil about 6 hours after food had been removed, in order to insure an acid-free condition of the pouch early the next day. Such pre-treatment with castor oil induces no change in character of the mucous secretion or its rate of flow. The following morning the animal was suspended in an adjustable harness and the contents of the pouch were collected, according to our usual technique. Unstimulated ('spontaneous') secretion was collected until its pH was above 6, i.e. until the absence of contamination by an appreciable amount of parietal secretion was established. The stimulus was then applied as previously described (2), and the secretion was collected for 4 successive 30-minute periods. Starting with the eugenol emulsion removed from the pouch, these specimens were labeled 1 to 5, respectively, this period of about 2½ hours of stimulation and collection constituting *cycle A*. At the end of the cycle, the dog was removed from the stand and allowed to rest for about 15 minutes before starting the next *cycle B* (or *C*, *D*, etc.) After every second cycle, the animal was allowed to drink water and to run free in the laboratory for 15 to 30 minutes; no food was offered until the end of the experiment.

The entire study on any one dog comprised a set of 3 experiments, arranged in the following sequence: *a*) The *fatigue experiment* proper, consisting of 6 or 7 cycles as outlined above (18–21 hours); *b*) a first recovery period of 30 to 36 hours, followed by the *first follow-up experiment*, usually lasting for 2 cycles (6 hours); *c*) a second recovery period of 3 to 5 months, followed by the *second follow-up experiment* of 3 or 4 cycles (9–12 hours). In the fatigue experiments the cycles were repeated until a persistent alteration developed in the visible character of the secretion. The number of cycles in the follow-up experiments was determined by the time necessary to attain essentially the same condition of the secretion as at termination of the fatigue experiment with the same dog. During recovery periods, the dog was kept in its cage and supplied with food and water as usual. No animal was used for more than one complete study. One dog died of a pouch perforation one week before the second follow-up experiment was scheduled.

All the specimens were examined separately from the physico-chemical and microscopic aspects. Results of the latter examination will be reported subsequently (8). The characteristics of the specimens included in the present report are: *1*) volume, *2*) pH, *3*) specific gravity for all but the most viscous specimens, *4*) per cent solids, *5*) coloration due to blood, *6*) opacity and *7*) viscosity. Volume was determined by collecting the specimens in graduated 15-ml. centrifuge tubes; pH by glass electrode; specific gravity by the falling-drop method; per cent solids was calculated from dry weight, after drying to constant weight in an oven at 100 to 105° C. Color of the secretion was evaluated by comparison with Munsell color charts (9), and recorded in their system of notation. In the final analysis of the data, only intensity of red was used (i.e. for blood), and this was rated on an arbitrary scale of 0 to 4 degrees. Opacity and viscosity were also quantified arbitrarily, on a scale of 5 degrees, by direct comparison with a set of graded standards designed especially for these experiments.

Opacity standards ranged from complete transparency (grade 1), through translucence and opalescence, to complete opacity (grade 5). Their composition is shown in table 1. They were prepared as follows: To a test tube containing the indicated volumes of $BaCl_2$ solution and water at room temperature, Na_2SO_4 solution is added slowly with constant agitation. Immediately thereafter, the warm filtered agar solution is added, and the mixture is shaken vigorously to achieve uniform distribution of the fine $BaSO_4$ precipitate. The suspension is rapidly transferred to a 5-cc. glass ampule by means of a capillary pipette or a syringe with a long needle, and the ampule is immediately set in an ice bath to congeal the agar mixture. Air bubbles, formed during the shaking, must be allowed

[6] Tergitol-Penetrant was kindly supplied by the Carbide and Carbon Chemicals Corporation, New York City.

to escape before the mixture solidifies. The ampule is sealed off rapidly, with care not to reliquefy the gel by heat, since a uniform gel of this character will not form again once it has been melted. Speed in handling the warm agar mixture throughout the preparation is of paramount importance, in order to keep the $BaSO_4$ uniformly suspended. The viscosity standards ranged from a fluid which flows almost as freely as water (grade 1) to a gel which hardly flows at all on inversion (grade 5). Their composition is given in table 2, and they were prepared as follows: A weighed portion of a solid polyvinyl alcohol (Elvanol, Du Pont Grade 91–65)[6] is introduced into a clean soft-glass test tube (150 x 16 mm.) and the water is added so as to wash down any solid material that adheres to the sides, without wetting the upper portion of the tube. The contents are well mixed with a glass rod, after which the tube is drawn out and sealed about one inch from the end. When cool, it is placed in a boiling water bath until complete solution of the solid is achieved. Water that condenses on the upper portion of the ampule should be brought back into the solution by shaking. Bubbles trapped in the viscous liquid usually disappear within 24 hours at room temperature; they may be removed more quickly by allowing the tubes to stand in hot water for 1 to 2 hours.

EXPERIMENTAL RESULTS

For analysis of the observations on any one animal, the data were plotted against specimen number (*A2*, *B4*, etc.) and the corresponding time, in a series of histograms, each line of which represents a single characteristic of the mucous secretion. An illustrative set of data for one dog is shown in figure 1, and reveals both intra- and inter-cyclic relations of all specimens from the fatigue and follow-up experiments. The first specimen in each cycle (*A1*, *B1*, etc.) has no significance because it consisted chiefly of eugenol emulsion; hence, they were not included in this or subsequent analyses. In order to study inter-cyclic relations as stimulation was repeated, the data for the 4 samples within each cycle were averaged and the arithmetical means plotted against the cycle designations (fig. 2). Since the responses in different dogs were reasonably uniform, these data for all 4 animals were averaged in the same way, cycle by cycle, and the means plotted in a composite graph analogous to those for the individual animal (fig. 2). In the following presentation of results, we have considered *1*) intra-cyclic variations in the fatigue and follow-up experiments; *2*) inter-cyclic relations within each of these experiments and also between them.

Response Within Any One Cycle

Volume. Remembering that small differences in volume/unit time are insignificant (2), it is seen from fig. 1 that the rate of secretion (i.e. volume/30 minutes) tends to decrease in the course of any one cycle. In about 60 per cent of the specimens, the greatest volume of secretion was collected during the first half-hour (*Specimen 2*) after removal of the eugenol stimulus; in most of the remainder, the peak rate came in the second half-hour. The cycles were never prolonged to the point where the rate of flow equalled that for spontaneous secretion. Based on early experience, however, the amount of secretion which would have been collected in a sixth specimen would have been too small to allow the requisite observations. In every case, the next application of eugenol for the subsequent cycle yielded a relatively large volume of secretion.

Opacity. This property seems to follow a general pattern, regardless of cycle or experiment. The first post-stimulus sample usually possesses the highest opacity

[6] Elvanol was kindly supplied by E. I. du Pont de Nemours and Company, New York City.

attained in the cycle; this occurred in over 80 per cent of the fatigue cycles. The next specimen may be equally opaque, but it is usually less so, and the fourth and fifth specimens are always the lowest. The peak opacity for any one cycle attained a rating of 5 in more than half (52%) of the cycles.

Viscosity. In general, viscosity is correlated fairly well with opacity within any one of the first 3 or 4 cycles of the fatigue experiment and the first 1 or 2 of the follow-up experiments. In the later cycles, however, correlation is poor, for an essentially constant viscosity of low grade may be accompanied by an opacity of almost any magnitude. The dual origin of the opacity suggested by this observation is discussed later. The peak viscosity was attained in *specimen 2* in 24 of the 25 fatigue cycles (96%), and in *specimen 3* only once (4%), as compared with the corresponding incidences of 84 per cent and 16 per cent, respectively, for opacity. It is only in *cycle A* that the viscosity attains a grade of 4 or 5, so high that the secretion is barely able to flow out of the tube when it is inverted, and a relatively high viscosity is maintained throughout this first cycle. Thereafter, in all of the subsequent samples of

TABLE 1. COMPOSITION OF OPACITY STANDARDS

REAGENTS	GRADE OF OPACITY				
	1	2	3	4	5
	Volumes in ml.				
$BaCl_2$ (0.2N)	0	1.0	1.0	1.0	1.0
H_2O (dist.)	2.0	1.0	0.9	0.8	0
Na_2SO_4 (0.25N)	0	0.02	0.08	0.2	1.0
Agar (2%)[1]	2.0	2.0	2.0	2.0	2.0

[1] Filtered while warm through Whatman filter paper #2.

cycles B et seq., regardless of whether they occur in a fatigue or a follow-up experiment, the viscosity never rises above a value of 2 and frequently stays at only grade 1.

Per cent solids. This also tends to be highest in the early half of each cycle. The magnitude of this maximal value varies considerably from cycle to cycle, and from experiment to experiment, a difference which is reflected in the cycle averages discussed later. The highest value observed for each dog varied from 5.5 to 6.0 per cent; the lowest from 2.0 to 2.5 per cent. Thus, the over-all variation was about 3-fold, from 2.0 to 6.0 gm/100 ml. In general, variations in per cent solids within any one cycle parallel those for volume/unit time and opacity, but the gradation is rather less orderly for the dry weight data.

Color. In contrast to the foregoing, the intensity of blood color tends to increase as secretion continues within any single cycle. At no time, however, is the coloration so deep that it indicates any considerable amount of frank bleeding. The last two specimens (*4* and *5*) are usually redder than those collected directly after removal of the stimulus, but even this is not invariably true. When the trend is different from this, it seems likely that the discrepancies arose accidentally from mechanical trauma at the neck of the pouch during administration of the stimulus.

pH. The pH reaches a maximum value above 8.8, and rarely falls below 7.4 for any of the specimens. The variation for the successive specimens of any one cycle show no uniform trend; sometimes there is a consistently progressive increase from beginning to end of the cycle; sometimes there is an equally consistent decrease; in other instances the variations are insignificant.

Specific gravity. In contradistinction to the other characteristics, the specific gravity undergoes but little change within any one cycle. In fact, except for a single questionable value of 1.030, the range throughout all the experiments was 1.012 to 1.022, and there was no suggestion of an intra-cyclic trend in any instance.

Mean Response From Cycle to Cycle

In order to study the changes in the characteristics of the mucous secretion which result from *repeated* application of the eugenol emulsion, in contrast with those just presented, which reflect the response to a *single* application, the arithmetical mean was calculated for each set of 4 values (*specimens 2–5*) within any one cycle. These averages were plotted against the cycle designation (fig. 2).

Fatigue experiments. As for intra-cyclic changes, individual responses of the 4 dogs show striking similarities, sufficient to warrant some tentative generalizations. For opacity, viscosity, and per cent solids, the curves drop after *cycle A*, the first two characteristics reaching a minimum about the third cycle, and per cent solids by the second. Following this drop, the viscosity curves level off, whereas those for opacity and per cent solids rise again to the end of the experiment, these changes being observable in almost all the individual curves as well as the composite ones. Terminal values for opacity are all less than the corresponding initial cycle values, in contrast to the data for per cent solids, in which terminal values equal or exceed those for *cycle A*. Individual curves for volume rise rapidly in the first half of the experiment, but this sharp rise does not continue after the third cycle. However, the composite curve for this variable suggests that the volume-rate of secretion is practically constant during the latter half of the fatigue experiment. Individual curves for color manifest no critical change in curvature, such as are shown by the other characteristics, but rather a general tendency to rise throughout the experiment. This is corroborated by the composite curve which attains its highest value in the last cycle. On the other hand, pH falls off throughout the experiment, though irregularly, and its composite curve approximates a straight line with a slight downward slope.

Thus, at the beginning of the fatigue experiment, the specimens consist *on the average* of a relatively small volume of an opaque jelly-like material, with high pH and high per cent solids. At the middle of the experiment, the 30-minute volumes are about 7 times as large as at the beginning, and the total solids are relatively low. The material is now a decidedly pink gelatinous fluid, tenacious, transparent or translucent, and of slightly lower pH. By the last cycle, volume and dry weight are both maximal but the resemblance to mucus is lost completely; on the contrary, the specimens are now free-flowing, red, and resemble most nearly inflammatory exudate. Hence, as a result of repeated irritation with eugenol, the gastric mucosa no longer responds with the secretion of opaque viscous mucus; the secretory apparatus has been fatigued or exhausted, and an inflammatory process has been initiated.

Independent evidence of such inflammation was obtained from 2 of the dogs by endoscopic examination of the pouch mucosa, using a peritoneoscope, at the ends of the fatigue and the first follow-up experiments. The mucosa was covered with a transparent layer of mucus, and petechiae were occasionally visible. However, there was no evidence of hemorrhage from any one vessel in either of these examinations.

First follow-up experiments. Thirty to 36 hours after completion of the fatigue experiment, the response of the mucosa is strikingly different. In general, average opacity, viscosity, pH and blood coloration in *cycle A* of the first follow-up experiment are about the same as in *cycles B* or *C* of the fatigue experiments; volume/unit time is intermediate between those of fatigue *cycles A* and *B*. Per cent solids and specific gravity are almost invariably lower than at any time in the course of the fatigue experiment. The mean characteristics of the secretion of *cycle B* of this follow-up experiment are generally typical of the material obtained about the middle of the fatigue experiment, the only notable exception being the lower per cent solid values.

These observations indicate that a 30- to 36-hour rest after 6 or 7 applications of eugenol was not sufficient to restore the gastric mucosa to its state prior to the initial application. Some restoration of function did occur, since the secretion obtained at the beginning of the follow-up experiment was unquestionably mucus of the kind collected before the middle of the fatigue experiment, but the restoration was far from complete, as evidenced by the rapidity with which the glands could be fatigued this second time.

TABLE 2. COMPOSITION OF VISCOSITY STANDARDS

GRADE NO.	'ELVANOL' mg.	WATER ml.
1	250	5
2	350	5
3	500	5
4	625	5
5	725	5

Second follow-up experiments. The response to the first application of eugenol 3 to 5 months later, with no experimental manipulation of the mucosa during this interval, is essentially the same as in *cycle A* of the fatigue experiment. *Cycles B* and *C* are in most cases similar to the third or later cycles of the fatigue experiment. Only in regard to blood content, which is increased throughout the second follow-up experiment, and possibly per cent solids, is there any clear difference. Thus it appears that secretory activity of the mucosa has been restored nearly to its original state. However, the ease of bleeding suggests that, at this stage of the study, the tissue is less resistant to irritation than it was before treatment with the eugenol. See below for further evidence in support of this.

DISCUSSION

In spite of the complexity of the physiological phenomena here investigated, the uniformity of the data from dog to dog is sufficient to demonstrate their essential significance. This is evident from a comparison of the 4 individual curves for any one characteristic of the secretion and their composite, and also from a comparison of the several composite curves for different characteristics.

Within any one cycle, the volume/unit time and dry weight diminish from beginning to end of the the cycle with considerable regularity. Such evidence for a slowing of secretion within the 3 hours following a single eugenol application can be observed in follow-up as well as fatigue experiments, and it is consistent with the simultaneous reduction in opacity and viscosity. Densely opaque and viscous mucus is obtained consistently only during the first hour after stimulation. During the second hour, viscosity and opacity both diminish radically, and by the middle of the third hour, flow rate has returned nearly to its basal level. Actually, the data on rate of collection of the secretion are not closely representative of its rate of formation, because of its high tenacity and viscosity. This is supported by the presence, post mortem, of a considerable amount of viscous mucus on the mucosal surface 15 minutes or

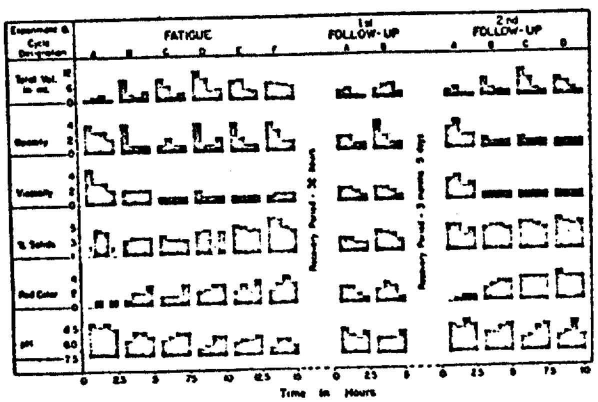

Fig. 1. Intra-cyclic variations in physical and chemical characteristics of mucous secretions induced by eugenol in a series of 3 experiments on one dog (*No. 175*), including the fatigue experiment (6 cycles), the first follow-up experiment (2 cycles), and the second follow-up experiment (4 cycles).

more after the end of a cycle of stimulation and collection. This implies that the output of mucus by the mucosa takes place more rapidly, and ceases sooner than is indicated by the volume-time curves themselves.

The specific gravity is constant within one per cent (1.012–1.022), whereas dry weight varies from 1.6 to 6.0 gm/100 ml. The decrease in per cent solids during the first 2 cycles of a fatigue experiment may be a consequence of the progressive diminution in the rate of mucin secreted. During the subsequent part of the experiment, however, there is an increased oozing of blood cells and plasma, and since both of these have a higher dry weight than mucus alone, their admixture results in the elevation in solid content. There is a notable lack of concordance between the dry weight and specific gravity throughout, but in view of the complex composition of these mixtures, and the hydrophilic character of the muco-proteins, this is not surprising.

Variations in intra-cyclic pH of 7.4 to 9.0 are consistent with our previous observations (3, 5). At one time, it was suspected that differences in pH might be indicative of significant differences in character of the specimens, but subsequent evidence, based on equilibration with alveolar air immediately before the pH determination, revealed that values above 7.4 (without equilibration) may be artifacts (10) resulting from loss of CO_2. The present high pH values may reflect differences in content or character of the buffer substances present, but this relation is still unknown.

The increase of blood toward the end of each cycle without evidence of gross bleeding from the mucosa indicates that irritative processes also come into play in these experiments, and that they persist beyond the termination of the secretory reaction. Observations of red and white cell content of these same specimens (8) lend further weight to the present evidence. This finding confirms other reports that what is currently called mucous secretion frequently contains transudate and exudate (2).

From these as well as previous eugenol experiments, it may be inferred that the actual mucus-secretory response of the mucosa to any *single* application of the irritant lasts for only a short time. However, cessation of secretion following a single application must not be confused with fatigue or exhaustion of the glands themselves. That we are dealing here with both phenomena is shown by the continuing, but diminishing, responsiveness of the pouch to successive applications of eugenol in a fatigue experiment. During this time, however, the average response for successive cycles undergoes a progressive transition from a material which is chiefly viscous mucus, to one which consists almost solely of transudate and bloody exudate. During the first 3 cycles this change is fairly rapid, and by the beginning of *cycle C*, the specimens are already clearly different from typical viscous mucus, as evidenced by the mean graphs for volume, opacity, viscosity and per cent solids. By the fifth cycle, the changes in these characteristics are virtually complete; the increase in blood content continues to the end of the fatigue experiment, and the specimens appear to consist predominantly of inflammatory exudate. The 7-fold average increase in volume/30-minute specimen in the course of the first half of the experiment is particularly striking in contrast with its leveling off beyond *cycle C*. This suggests that the analogous inter-cyclic leveling off of viscosity is chiefly the result of dilution by a non-mucoid (e.g. interstitial) fluid, whereas the intra-cyclic reduction in viscosity, which is accompanied by a *reduction* in volume, results from a falling off in mucin-containing secretion. It is significant that the opacity and viscosity curves for the fatigue experiments both drop to a minimum about the third cycle, but the former then rises to the end of the experiment, whereas the latter remains stationary. Were the opacity a consequence of its mucous cell content predominantly, this difference between the 2 curves would not be understandable. However, mucous cells and cell residues constitute only one of the major components contributing to opacity; another is white blood cells, and a third is coagulated mucin. It is probably one or both of the latter that accounts for the progressive increase in opacity during the last 3 or 4 cycles. As for the variations in pH from cycle to cycle, all 3 composite curves show a slight downward trend within a range of about half a pH-unit, but there is no critical change in curvature, and no particular significance need be attached to these results at present.

Hence, the response of the gastric mucosa to a single stimulation with eugenol emulsion results from a combination of mucigogue, desquamatory and mildly inflammatory actions. With the continuing desquamation which results from its repeated application, the mucosa undergoes extensive exhaustion of its mucus-secreting apparatus. At first, such a chemical abrasion or denudation of the mucous glands results in a mild inflammatory process, constituting another, less specific, defense reaction. The resultant increase in transudation accounts for the increase in volume and therefore for the dilution of the mucus which is observed even during the first 3 cycles. As the desquamatory process extends deeper into the mucosal layer, blood capillaries are exposed and ruptured. By the sixth application of eugenol, mucus-secreting cells appear to have been removed in considerable degree, and those which remain must have been emptied of their intra-cellular secretion. The secretion of this stage of the experiment is in many respects similar to that obtained after

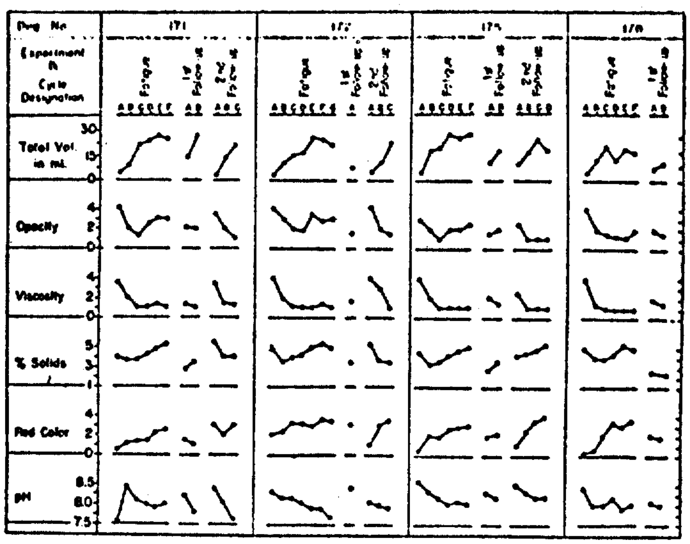

Fig. 2. Comparison of graphs of inter-cyclic variations in the physical and chemical characteristics of mucous secretions induced by eugenol in experiments on dogs. Each point represents averaged data for all specimens of any one cycle.

irritation of the mucosa with mustard oil (4), i.e. large volumes of relatively transparent material, with low viscosity, and essentially no columnar cells.

How much time is required to effect a regeneration of the mucous barrier in the dog? The first follow-up experiments indicate that 36 hours after the final application of the eugenol in a fatigue experiment, the mucosal surface has already been reconstituted in great part, and is capable of renewed secretory activity. The character of the secretion, however, is not exactly the same at the beginning of this follow-up experiment as it was at the beginning of the fatigue experiment, 1.5 days previously. Mucus content was lower and content of transudate higher than in the latter, indicating that recovery is far from being complete. Further evidence for this is derived from the finding that the tissue can now be exposed to only about 2 applications of the desquamating agent before it attains the state of exhaustion for which 4 or 5 applications were required initially.

The second follow-up experiments demonstrate that functional recovery 3 or more months later was appreciably more complete than at the time of the first follow-up experiments, for the characteristics of the specimens from *cycle A* are now essentially the same as after the very first treatment with eugenol. A relatively greater blood content, however, suggests that reconstitution of the mucosal layer may still be incomplete, and that resistance to irritation is not yet at the level of the wholly untreated tissue. This is supported by the microscopic study of these specimens (8). A more complete investigation of these regenerative processes of the gastric mucosa, entailing histological studies of the tissue taken post mortem in the course of these and similar experiments, is nearing completion, and a preliminary report has already been published (11).

CONCLUSIONS

The mucus collected from the gastric (corpus) mucosa of Heidenhain pouch dogs following a single application of 5 per cent aqueous eugenol emulsion for 15 minutes is opaque, jelly-like and whitish, and contains only slight amounts of fluid suggestive of an inflammatory reaction. Successive applications of the stimulus, at intervals of about 3 hours, result in a progressive diminution in amount of mucus secreted, but there is a gradual increase in the quantity of a clear, free-flowing, sero-sanguinous fluid mixed with the viscous material. The proportion of the non-viscous component becomes considerable only after 3 or 4 such applications, and by the fifth or sixth, the material is entirely serous and virtually free of true mucus. From this, it is evident that the mucus-secreting function of the dog's pouch is seriously impaired by 3 to 4 15-minute treatments with eugenol under the experimental conditions employed in this study, and that no more than 5 or 6 such applications are required for its fatigue or complete exhaustion. Function is quickly restored, for 36 hours after the end of such a prolonged experiment the mucosa gives appreciable evidence of renewed mucus-secreting activity. Recovery is not very extensive, however, for the secretion now assumes a sero-sanguinous character much sooner than it did in the original fatigue experiment. In fact, only 2 cycles of eugenol stimulation are now required to attain the same degree of functional loss for which 4 or 5 cycles were originally needed. After the pouch mucosa had been permitted to go without any such treatment for 3 to 5 months, a third series of eugenol applications showed that mucus-secretory function had been restored almost to its original state. Only in respect to ease of bleeding does the tissue still give any evidence of the drastic treatment to which it had been subjected in the initial fatigue experiment.

Thus, a set of experimental conditions has been defined for the exhaustion of mucus-secreting activity in canine gastric pouches. This process includes a loss not only of cellular function but also of cells themselves, from which it may be inferred that the mucous barrier has been seriously impaired and perhaps even completely destroyed. Within one and a half days following such destruction, the barrier may be reconstructed in appreciable degree. However, even 3 months later there is still evidence that its condition may not yet be entirely normal, although secretory power appears to be fully restored.

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On the effect of some essential oils on the motor state of the intestine

by Yoshio Sone and Shiro Hitati

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Gunn (1) found a reduction of the tonus and an inhibition of the peristaltic movements of the intestine when introducing various essential oils into the lumen of the intestine of rabbits, and he concluded that the carminative effect of the above substances is due to these actions. In contrast, Plant (2) observed in dogs which received the essential oils in the intestine an increase in tonus and an accelerated peristaltic movement, which he contributed to their carminative action. It is readily understandable that flatulence of a spastic nature is eliminated by substances, like ginger, which has an intestine-inhibiting effect, while the frequently occurring paretic flatulences can only be treated with peristaltic-improving substances. It is therefore very likely that the essential oils, which are frequently used successfully in the treatment of paretic flatulence, have a peristaltic-improving effect on the intestine, as it was found by Plant. What then is the cause of the inhibiting effect of the above mentioned substances observed by Gunn? It may be perhaps the type of test-animal be used. At any rate it is desirable to decide whether the intestine of the rabbit behaves in the same way against essential oils as that of dogs. For this reason tests were made on the excised intestine of the rabbit with some essential oils, like cinnamon-, clove-and fennel oil, which were also investigated by Gunn.

Tests on the intestine in situ

Rabbits of about 2 kg body weight were secured in the dorsal position. Under ether narcosis the abdominal cavity was opened and the apparatus indicated by one of us (3) set to introduce the liquids to be tested into the lumen of a small intestine section to be able to record its tonus and peristaltic movements. The essential oils were produced by means of gum arabic and Ringer solution in emulsions of 1:100, diluted in use with a gum-containing Ringer solution to any desired concentration, and heated to body temperature.

Cinnamon oil effected during the introduction in an emulsion of 1:100 to 1:1000 into the intestine lumen an increase of the tonus and an increase of the peristaltic movements both in frequency and extent, which occurred practically immediately after the administration and which attained their maximum after a few seconds and remained at this level for a long time, as it can be seen from fig. 1. These changes of condition subsided gradually when the emulsion was rinsed out with Ringer solution. With an emulsion of 1:2000 there was a slight but clear acceleration and intensification of the peristaltic movements with a very insignificant tonus increase.

Fig. 1 Small intestine in situ of the rabbit. During the signal an emulsion of 1:100 was introduced into the intestine. Between 1 and 2 an interval of about 30 minutes

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Fennel oil produced the same changes of condition of the intestine, which were less conspicuous, however, than with cinnamon oil. Clover oil showed a similar behavior as fennel oil, but was far weaker than the latter, so that it could only produce an acceleration and intensification of the peristaltic movements in a 1% emulsion, but no significant tonus increase.

This result is in very good agreement with Plant and indicates that the intestine of the rabbit behaves toward essential oils just like that of the dog. Why Gunn found only an inhibiting effect of the said substances is thus still an open question. But there is no doubt that the favorable result of the essential oils, at least of those tested, depends on their enhancing effect on the intestine.

Tests on the excised intestine

In the test on the excised intestine of the dog, of the cat and of the rabbit, Muirhead and Gerald (4) found that different essential oils produced a tonus increase in addition to an acceleration of the peristaltic movements, while Gunn (1) achieved a tonus decrease and an inhibition of the movements in the test on the excised intestine of the rabbit with essential oils, some of which had been tested by the first mentioned researchers. The reason for this strange difference could not be determined yet. But it could be assumed that it may be due to the different concentrations of the essential oils tested by the above mentioned researchers, because mustart oil, which causes in high concentrations a marked decrease of the tonus and an inhibition of the movements, also effects in low concentrations a definite tonus increase and acceleration of the movements according to the investigation by one of us (3). In order to answer this question, we made tests on the excised intestine of the rabbit with cinnamon-, fennel- and clover oil, which had been investigated by Gunn also.

The minimum concentration in which the cinnamon oil emulsion could produce a marked change of condition in the excised intestine was 1:10,000,000. In this concentration cinnamon oil effects a slight acceleration and intensification of the peristaltic movements. This change was very conspicuous with a concentration of 1: 1,000,000 and was accompanied by a marked tonus increase, as it can be seen from fig. 2. With a concentration of 1:100,000 the same changes occurred, but they turned sooner or later into the opposite. With 1:10,000 there was no tonus increase and an intensification of the movements, but right from the beginning a decrease of the tonus and inhibition of the movements which put the intestine after a short time into complete relaxation and rest, as it can be seen from fig. 3.

Fig. 2 Excized small intestine of rabbit. At the signal 0.1 cc of a 0.1% cinnamon-oil emulsion introduced into the bath of 100 cc.

Fig.3 Excised small intestine of rabbit. At the signal 0.1 cc of a 1% cinnamon-oil emulsion introduced into the bath of 100 cm.

Fennel-and clover oilcaused similar changes of condition, but were very far in their effectiveness behind cinnamon oil.

As expected, the essential oils have in low concentrations a hypertonic and peristalsis-increasing effect, but in high concentrations a hypotonic and peristalsis-inhibiting effect. It is likely that the changes of condition caused in the intestine in situ of the rabbit by the said substances are substantially the same as those caused in the isolated intestine by the same substances in low concentrations. But the question is why the essential oils, which only have an enhancing effect on the intestine when they are introduced into the lumen in situ in concentrations, like 1:100 to 1:1000, have the same effect on the excised intestine in concentrations like 1:1,000,000 and an opposite effect in concentrations like 1:100,000. This is perhaps due to the fact that the essential oils can enter the intestinal tissue easier and faster from the serous skin than from the mucous membrane. In order to determine whether this assumption is correct, we made the following test:

An excised rabbit intestine of about 7 cm length was secured on the apparatus which was used in the test on the intestine in situ by introducing its two canules into the two incisions of the intestine. After connecting the head of the intestine to a holder secured on the apparatus, and the oppositepoint with a thread which was later to be connected to a recording lever, the intestine was introduced into a hot Ringer bath. In order to allow the liquid to be tested to act on the intestine from the mucous membrane, it was introduced into a funnel connected to one canule. But when we wanted it to act on the intestine from the serous skin, it was added to the Ringer bath. The concentration of the cinnamon oil emulsion, which could produce a marked change of condition when introduced into the lumen of the intestine, was 1:1000. The change of condition consists, as in the intestine in situ, in a tonus increase and acceleration of the peristalsis, When added to the bath, cinnamon oil effected in a concentration of 1:10,000,000 an acceleration of the peristaltic movements, in a concentration of 1:1,000,000 in addition also a tonus increase, but in a concentration of 1:10,000 a great decrease of tonicity, and the peristalsis stopped. The result namely that the enhancing phenomenon is produced in the intestine with cinnamon oil on the serous skin in a much lower concentration than on the mucous membrane, seems to be accounted for, as expected, by the fact that cinnamon oil can penetrate much easier into the tissue from the serous skin than from the mucous membrane. That cinnamon oil causes no inhibiting effect when introduced into the intestine in situ ina concentration like 1:100, seems to be due mainly to the same reason, though the elimination of cinnamon oil by the circulating juices may also play a part here.

An isolated intestine ssspended in the Ringer bath, whose vagus end had been paralyzed by treatment with atropine, responded to the addition of cinnamon oil with a tonus increase and accelerated peristalsis, just like the unatropinized intestine. When an excised intestine had been treated first with magnesium, it reacted to the addition of cinnamon oil neither with a tonus increase nor with accelerated peristalis, though a considerable tonus increase could be produced by the addition of barium, as it can be seen from fig. 4. The tonus increase and peristaltic acceleration caused by cinnamon oil is thus caused by the excitation of the automation system of the intestine, though it is not clear whether this excitation is the result of a direct or indirect action.

Summary

Cinnamon-, clover and fennel oil, when introduced into the intestine in situ of the rabbit, produces in low concentrations an acceleration and intensification of the peristaltic movements and in somewhat higher concentrations also an increase in tonicity.

In the excised intestine of the rabbit suspended in the Ringer bath, they cause the same changes of condition, but in far lower concentrations than in the intestine in situ. This is mainly due to the fact that they can enter much easier from the serous skin into the intestinal tissue than from the mucous membrane.

The favorable result of the said substances in the treatment of flatulence is due to the above described effect, but not to an inhibiting effect, which is

observed in the excised intestine and only in very high concentrations of the substances.

(Translated by Carl Demrick Associates,Inc/IE/t)

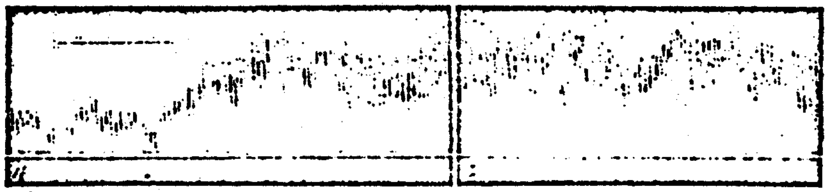

Fig 1

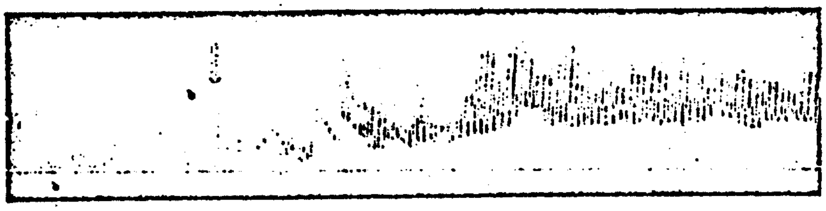

Fig 2

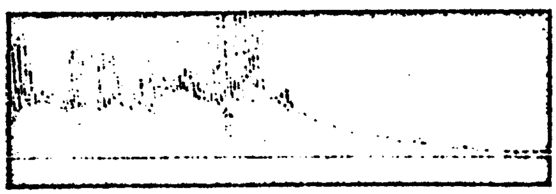

Fig. 3

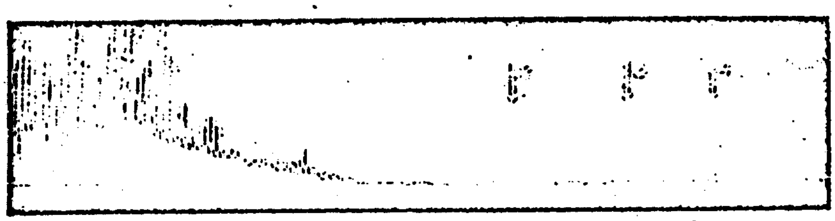

Fig. 4

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VARIATIONS IN CELL TYPES AND CELL DENSITY OF GASTRIC SECRETIONS IN DOGS FOLLOWING REPEATED EUGENOL STIMULATION*†

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It is the purpose of this report to present in detail the microscopical observations made upon successive secretion samples from Heidenhain pouch dogs following repeated stimulation of the mucosa with a relatively mild irritant. The irritant employed throughout all of the experiments was eugenol (4-allyl-2-methoxyphenol, Merck, U.S.P.). The experiments were designed to study the effects of repeated insult to gastric glandular epithelium in order to note (a) whether the mucus secretory processes could be fatigued or even exhausted, (b) the extent of morphological impairment of the gastric wall and (c) the degree of recovery of function after various lapses of time.

A complete description of the general experimental procedures has been elucidated in a previous paper,[12] in which the physico-chemical properties of successive specimens collected from the pouch were evaluated for the following variables: Volume, pH, specific gravity, per cent solids, opacity, viscosity, and intensity of blood color. For the present study, the same samples of mucus were utilized concurrently for an analysis of the variations in cellular contents. The meaning of several terms employed in these experiments may be briefly restated. After the eugenol emulsion, which served as stimulating fluid, had been removed from the pouch (having been kept there for 15 minutes), specimens were collected for 4 successive half-hour intervals. The combined period of stimulation ($\frac{1}{4}$ hour) and of collection (4 half-hour periods) was designated as one "cycle". A "fatigue experiment" consisted of 6 or 7 such cycles in succession, designated A, B, C, etc. Thirty to 36 hours after termination of a fatigue experiment, the pouch was exposed again to 2 cycles of stimulation and collection; this treatment was termed the "first follow-up experiment". Finally, after a 3–5 months recovery period, a "second follow-up experiment", consisting of 3 or 4 similar cycles, was inaugurated.

In all of the experiments on any one dog, of which 5 were used, about 60 secretion samples were gathered for study. Smears were prepared from each of these and then stained with toluidine blue after the method of Hess and

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† Preliminary reports of this work were presented before the Third National Gastric Cancer Conference held in Chicago, December 5–6, 1946,[9] and before the American Physiological Society in 1947.[13]

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Hollander.[8] Several of the components of the samples—e.g., columnar cells, mucous neck cells, parietal cells, leucocytes, and mucin (the latter estimated from intra- and extra-cellular metachromatic substances)—were quantitated on an arbitrary scale of zero to four-plus. Not only was the presence or absence of the components observed, but the structure and appearance of the cells was noted. As the material from the several animals was studied, it soon became apparent that the morphological variations within and between cycles occurred with relative consistency. A number of photomicrographs (which unfortunately cannot be presented in their original colors) will be discussed at appropriate places to indicate some of the alterations which regularly occurred in the course of the fatigue and follow-up experiments.

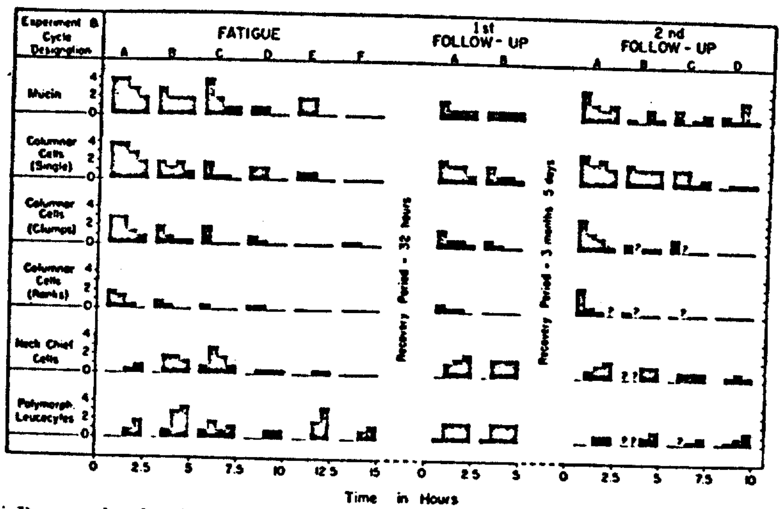

FIG. 1. Bar graphs showing the intra-cyclic variations in cell types and cell density of mucous secretions induced by eugenol in a series of 3 experiments on one dog (#175), including the fatigue experiment (6 cycles), the first follow-up experiment (2 cycles), and the second follow-up experiment (4 cycles).

OBSERVATIONS

The Fatigue Experiments

We shall set down the responses of a single dog in the course of an entire experimental sequence, cycle by cycle. The quantitative data for a typical experiment (Dog #175) are presented in a series of bar graphs in Figure 1. Within any one cycle, the sample-by-sample variations of each characteristic are depicted. The responses of the different dogs are compared in Figure 2. At the start of each experiment, spontaneous secretion was collected continuously and the first cycle was not started until this secretion became acid-free, with a pH of 5 or higher. Smears of such spontaneous secretion invariably contained no normal cellular elements, though occasionally cellular debris was evident.

Cycle A—The initial specimen (#1) obtained in any cycle was the eugenol emul-

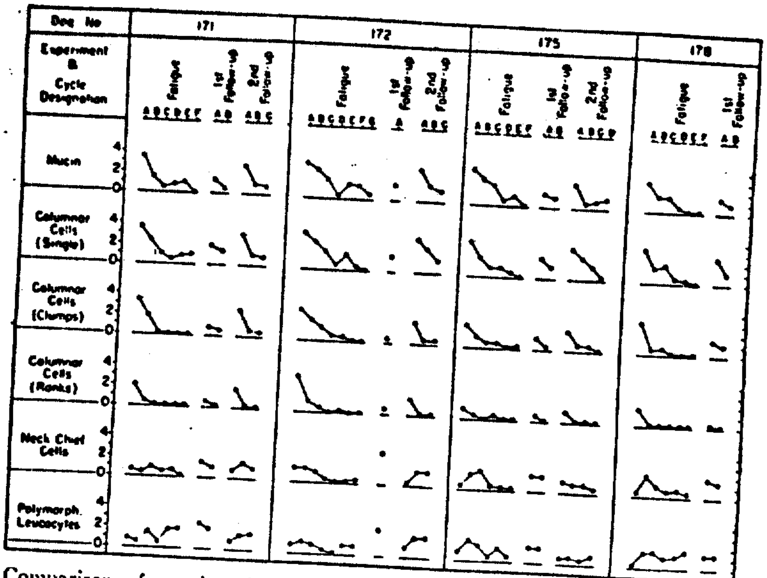

FIG. 2. Comparison of graphs of inter-cyclic variations in the cytological characteristics of mucous secretions induced by eugenol in experiments on dogs. Each point represents the averaged data for all the specimens of any one cycle.

FIG. 3. Smear of material taken from the eugenol stimulating fluid following its removal from the pouch during a first follow-up experiment (cycle A, sample 1). Vacuolated substrate; large numbers of poorly preserved columnar cells with intensely staining nuclei. 70X.

sion which had been applied as the stimulant. Columnar cells, poorly preserved, were evident in most of these eugenol specimens (Fig. 3). The cytoplasm was vacuolated, the nuclei were usually large and swollen, but occasionally shrunken, and stained intensely (pycnotic). The cells were triangular and short, not long and tapering as were those to be described in the initial post-eugenol samples. Occasionally, a small rank of columnar cells was noted. Cells were rarely dispersed freely in the medium, and were seen only within the confines of bits of clotted matter. Such masses, apart from the cellular elements, stained purplish, indicating the presence of mucin in these specimens, and they were characteristically vacuolated. The liquid portion of the eugenol was stained light blue, and never metachromatically.

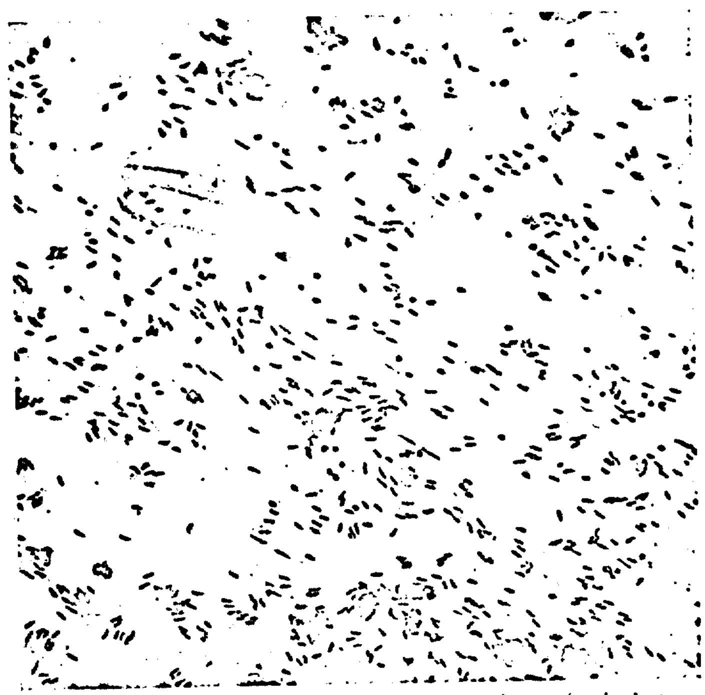

Fig. 4. Columnar cells in the initial cycle of a fatigue experiment (cycle A, sample 3). Masses of well-preserved, high columnar type of cells. Clumps of cells, single cells and occasional ranks may be noted in this picture. 70×.

The remainder of the cycle consisted of 4 half-hour specimens (# 2–5). The characteristic feature of the first two of these specimens was the presence of vast numbers of well-preserved columnar epithelial cells (Figs. 4 and 5). These appeared either as single elements, or in organized ranks of aligned cells, or in disorganized clumps. It is likely that some of the scattered single columnar cells were elements dissociated mechanically from the clumps and ranks during smear preparation. The columnar cell, as evident in a smear, was strikingly different in appearance from that seen in a tissue preparation. They were long, slender, and tapering, with an ovoid nucleus situated approximately $\frac{1}{3}$ to $\frac{1}{2}$ the distance from the apical end. In the cells of these early samples, the thecal cavity occasionally showed intracellular mucin. The columnar cell ranks might contain few or many cells; sometimes 50 to 60 columnar cells have been seen attached to each other laterally at the thecal region, but with the tapering tail-like portions separate one from another. The substrate in which the cells were embedded was relatively homogeneous, and gave evidence of the presence of mucin when stained.

In specimen 4, the secretion contained two types of material—one decidedly viscous, the other more free-flowing. Separate smear preparations were made of each type of secretory product. The thicker material contained well-preserved columnar cells, with a morphology as described above. The cells of the less viscous material showed a markedly different picture; their cytoplasm was frequently barely visible—sometimes not at all—and their nuclei were well preserved but seemingly naked

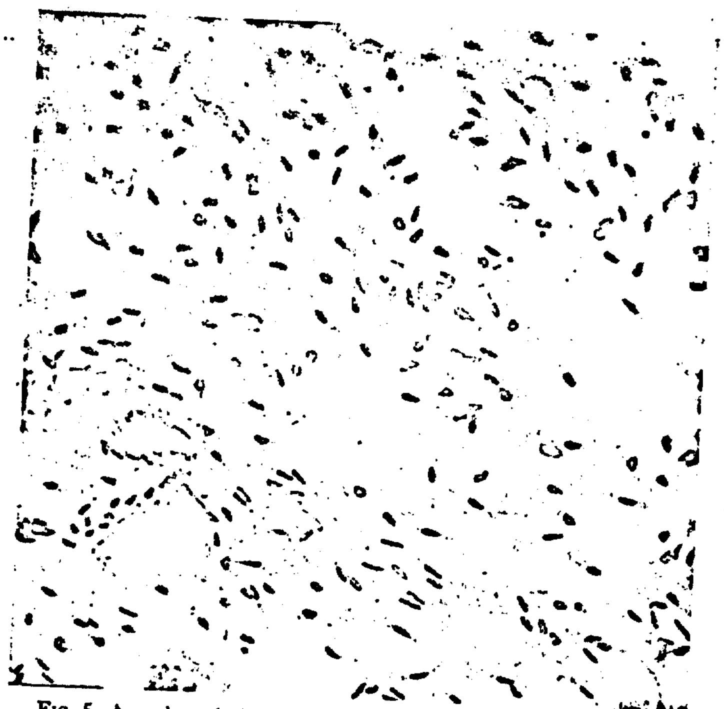

FIG. 5. An enlarged view of a portion of the field in Fig. 4. 310×.

without any visible cytoplasmic cover (i.e., ghosts). Other cellular elements, such as mucous neck chief cells and polymorphonuclear leucocytes (hereafter designated "polys") were noted at this time, primarily in the less viscous material. The neck chief cells were typically spheroidal. The mucin of their cytoplasm stained metachromatically with an intensity much greater than that evidenced in the thecae of the columnar cells. The nuclei of the neck cells stained dark blue and were usually flattened against one side of the spheroid (Fig. 6). Sometimes, however, the nuclei were seen in profile as small concave cups, giving the neck cell the appearance of an intestinal goblet cell.

By the end of the intial cycle of the fatigue experiment, the picture had become consistently one of poorly preserved columnar cells, some of which were only nuclei or nuclear remnants. The other components were large, well-preserved masses of leucocytes enmeshed in a fibrous substratum, and groups of mucous neck cells usually showing metachromatic cytoplasm and pycnotic nuclei.

Cycle B—As in Cycle A, the eugenol stimulating fluid contained some clotted masses in which were observed columnar cells similar to those described for the corresponding specimen of the previous cycle. The substrate containing the cells stained purplish in some regions; but different from the initial eugenol wash, other areas now stained light to dark green. From a mass of evidence in tissues as well as smears, we have come to regard the amorphous material which stains green as blood. These tinctorial indications of blood in the smears, when arbitrarily quantitated,

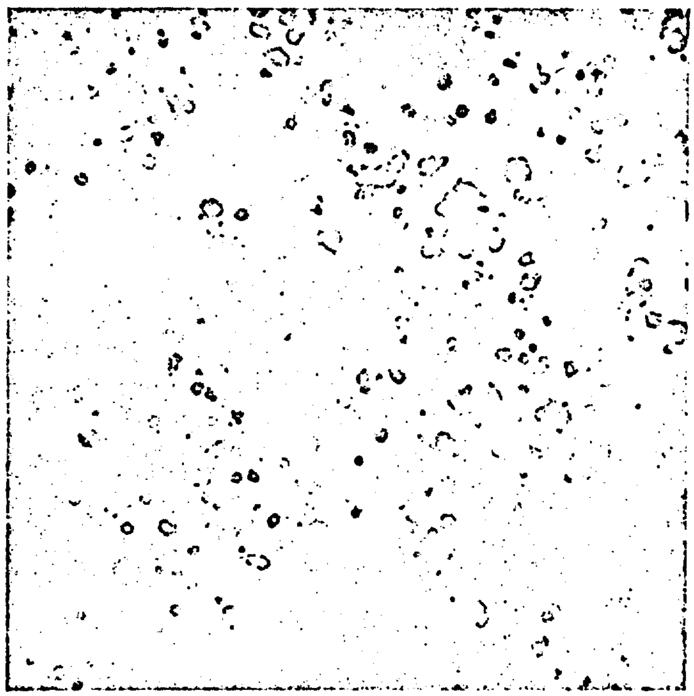

FIG. 6. Cells in this smear from a fatigue experiment (cycle C, sample 3) are primarily mucous neck cells. Purple metachromatic color has been lost. An occasional columnar cell and several erythrocytes are also evident. 310×.

parallelled closely the gross analyses of blood color intensity as reported by Sober, Hollander, and Sonnenblick.[12] Erythrocytes within the blood vessels also stained yellowish green with toluidine blue.

In the four post-eugenol specimens of this cycle, columnar cells were observed in extraordinarily large numbers, as single scattered cells and as clumps and ranks. This was especially so in the first 2 of the 30-minute specimens. By the end of cycle B, the number of these cells was considerably diminished, particularly for ranks and clumps. Most of the columnar cells, even those obtained in the early specimens of this cycle, were no longer of the high columnar type, but were low and triangular with the thecal end very wide. When one considers the huge masses of desquamated tall columnar cells evident in the smears of the initial cycle (Figs. 4 and 5), it seems possible that these low columnar cells are not from the surface of the mucosa *per se*, but rather from regions within the gastric crypts. Naked nuclei and remnants of columnar cells are seen with some frequency.

Large numbers of mucous neck cells and polys appeared in cycle B, and in 2 of the experiments this occurred even in the first cycle. For the most part, the neck cells showed intracellular metachromasia, but an occasional cell appeared empty of purple granules. In the last 2 half-hour specimens of the second cycle, the predominant cell types were neck chief cells and polys, while columnar cells were markedly diminished

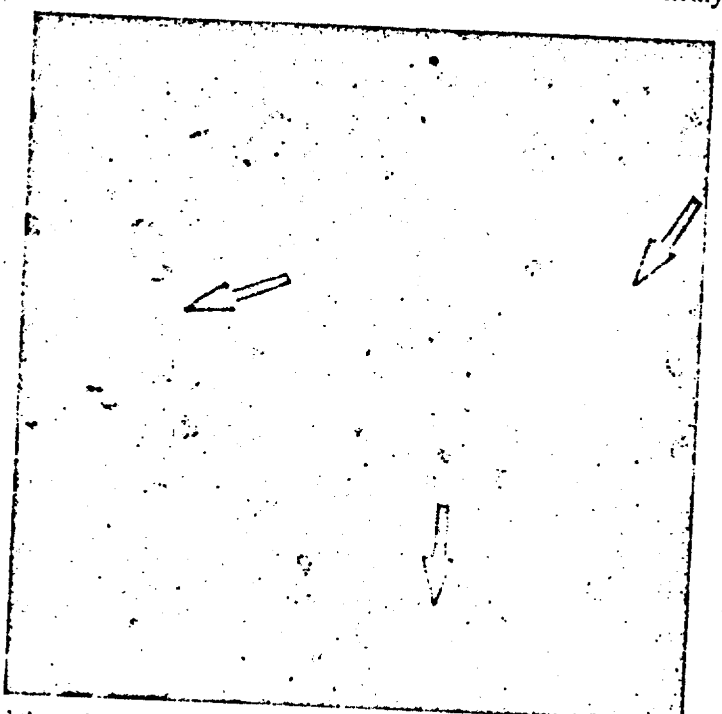

FIG. 7. Lightly staining parietal cells, of which three are binucleate (indicated by arrows), appear in this picture from a fatigue experiment (cycle A, sample 5). The field also includes at least six parietal cells with single nuclei. 310×.

in number. One still could observe, however, areas with badly preserved columnar cells, naked nuclei, and cellular detritus. Such cellular debris is evident in small greenish-staining bloody fragments.

During the course of this cycle another cellular element was observed in every experiment, i.e., parietal cells (Fig. 7), which were difficult to see because of their lightly stained greenish yellow cytoplasm. These cells were seen even in the initial cycle in 2 of the experiments. They were decidedly larger than neck cells or leucocytes, generally spherical or ovoid, and contained one or two round nuclei centrally placed. The parietal cells never stained metachromatically, as occurred with the mucous neck cells and, occasionally, the columnar cells. Because of differences in cell size, staining capacity, shape, and location and number of nuclei, it is possible to differentiate with no uncertainty between the parietal and neck chief cells. The former never appeared in large numbers in any specimen, and were generally difficult to locate without diligent microscopical search, even by an experienced observer.

Cycle C—The eugenol wash contained the same type of cell and substances as described for the previous cycles.

The first of the post-eugenol specimens were heterogeneous mixtures of materials of both high and low viscosity, such as generally appeared only in the latter half of cycles A and B. The highly viscous material contained large numbers of columnar cells, many of which were of high columnar type, and but few of which showed intracellular metachromasia. The more fluid part of the secretion contained decidedly fewer columnar cells, chiefly low, and these were poorly preserved with disrupted cell membranes. Polys and metachromatic mucous neck cells appeared in this less viscous portion. Thus, by the third cycle, neck chief cells, either separate or in clusters, appear as early as the initial post-eugenol specimen (Fig. 1). Smaller cells, about one-half of the size of the average mucous neck cell, were also seen during this cycle. They stained metachromatically, and had a nucleus which looked like, stained like, and appeared to be equal in size to, that of typical neck cells. During the 3 remaining 30-minute collection periods, large numbers of mucous neck cells were drained out of the pouch. Columnar cells were sparse, scattered throughout the smears, and usually poorly preserved (Fig. 8; cf. Fig. 5). By the end of this cycle, the predominant cell types were mucous neck cells and polys.

Cycle D—The eugenol drainage fluid contained only occasional patches of amorphous material, with few columnar cells and cellular remnants.

The succeeding samples of the fourth cycle were characterized by a marked diminution in their content of metachromatic material—in contrast with the previous cycles. Little purplish staining material was found in the substratum of any of the preparations, and the number of columnar cells was decidedly reduced. There was a paucity of single columnar cells as well as clumps, and organized ranks virtually disappeared. Evidently, the irritant emulsion no longer produced the exfoliation seen in Fig. 4.

No intracellular metachromasia was apparent in the columnar cells noted in any of the specimens of this cycle. These cells, even in the first post-eugenol specimen, were no longer high columnar in type; in general, they were not well preserved, and it was difficult to discern their cell membranes. The few remaining intact cells were more compact, and tended toward the cuboidal rather than tall columnar variety. These may be cells removed from the deeper portion of gastric crypts, where cells of this description are evident in tissue sections. A few parietal cells could be noted in the majority of the preparations. Clusters of very small round or polygonal cells, each with a minute cytoplasmic covering around a central nucleus, were also seen in this cycle. It was not possible to identify them with any morphological element of the gastric glands or blood. Throughout the fourth cycle, the content of mucous neck chief cells and polys was strikingly low and this was evident in every one of the experiments.

Cycle E—In all of the experiments the eugenol drainage fluid of the fifth cycle was found to have the typical lightly staining, fibrous, vacuolated substratum with occasional metachromatic patches. The interesting feature was the absence of any cellular components.

Preparations of the succeeding samples of this cycle showed a diffuse green color, indicative of blood, in many areas. These samples revealed blood macroscopically as well. The early part of cycle E was strikingly different from the early samples of A. The substratum was a dense fibrous mass, greenish in many areas with occasional small metachromatic patches. A few scattered single columnar cells were evident, but no ranks or clumps. Occasional well-preserved, delicately-stained parietal cells were noted. Neck chief cells and leucocytes were few in number during the early part of the cycle. Toward the end of the fifth cycle, the substratum was fibrous, less dense, and only a rare metachromatic area was evident. There was a decided paucity of columnar cells (the majority of these had evidently undergone cytolysis), but a moderate number of neck chief cells and huge masses of well preserved polys were present. The polys were the predominant and characteristic cell type in the final collection of this cycle. These were noted often as compact large aggregates in a fibrin mesh, indicative of inflammatory exudate (Fig. 13).

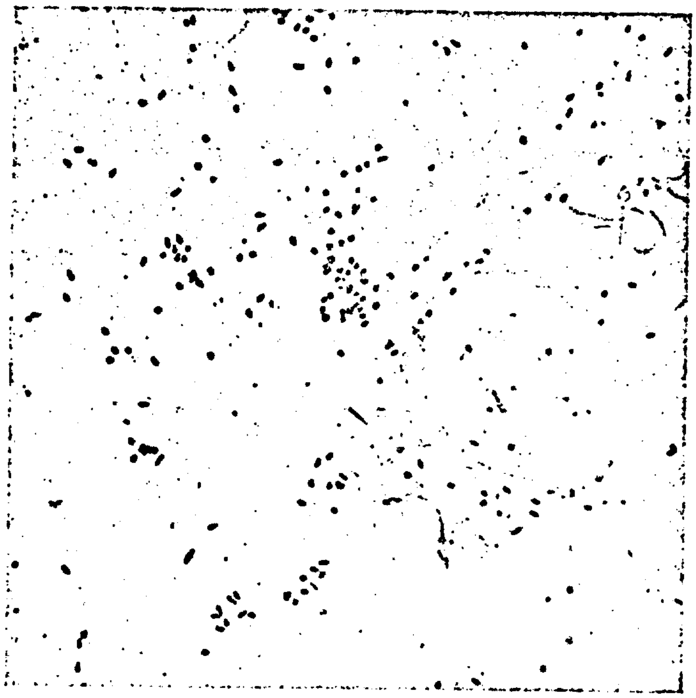

FIG. 8. Poorly preserved columnar cells and cell remnants in material from the third cycle, approximately the middle, of a fatigue experiment (cycle C, sample 2). Cells are fewer in number than in specimens from the earlier cycles (cf. Figs. 4 and 5), with cellular membranes typically disrupted, leaving naked nuclei. 70X.

Cycle F—All of the animals used in this investigation were put through 6 consecutive cycles of stimulation and collection, with the exception of one dog (# 172) which was given 7 cycles. The observations of this seventh cycle were virtually identical with those of the sixth, and therefore merit no separate description. The eugenol wash from the sixth cycle was invariably like that of the previous cycle, and was characterized by the absence of cells of any type.

The specimens of the entire terminal cycle were distinguished by a striking diminution in incidence of cells of all kinds, with the exception of leucocytes (Fig. 9). Intact columnar cells, either single or in other configurations, were a rarity, even

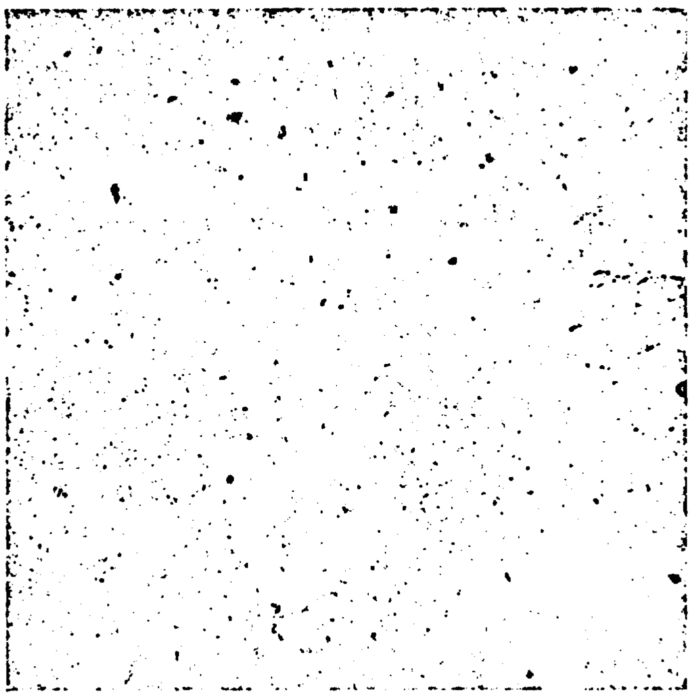

FIG. 9. Smear of secretion obtained during the last cycle of a fatigue experiment (cycle G, sample 3). Essentially non-cellular substratum evident. Columnar cells are absent, but large amounts of leucocytes may be observed in other fields at this stage. Cf. Figs. 5 and 8, which are from the beginning and middle of a fatigue experiment, respectively. 70×.

though additional preparations were stained and studied for confirmation. Some disorganized, poorly preserved columnar cells were evident in the early collection periods, and an occasional parietal cell and very few neck cells could also be noted. In no experiments were there recorded for the last 2 half-hour collection periods any observations of columnar cells in any form. A moderate to large amount of leucocytes was found in a fibrous network.

THE FIRST FOLLOW-UP EXPERIMENTS.

Following termination of the fatigue experiment, each animal was fed and rested for 30–36 hours, and then the first follow-up experiment was started.

This consisted of 2 successive cycles of stimulation and collection (only 1 in dog #172) like those already described. This number of cycles was determined by the time necessary to obtain specimens with essentially the same gross physico-chemical characteristics as those encountered at, nor near the end of the corresponding fatigue experiment.[12]

Cycle A (first follow-up): The eugenol stimulating fluid contained macroscopically visible masses of tissue-like material, and it is only in these that large numbers of columnar cells were again evident. The cells were wide and short, without the typica

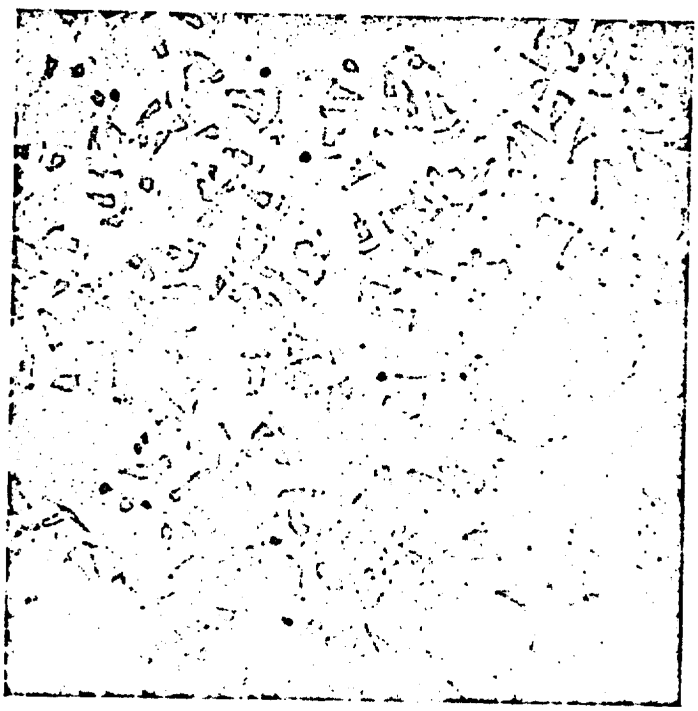

FIG. 10. Cells in eugenol stimulating fluid of first follow-up experiment (cycle A, sample 1). Columnar cells are short, thick, peculiarly vacuolated, and their nuclei stain deeply. 310×.

long tails of high columnar cells. The nuclei were pycnotic and often swollen, and the cytoplasm was vacuolated (Fig. 10). The masses, containing the cells, stained purplish, whereas the virtually cell-free substrate was light blue.

In the 4 half-hour samples, columnar cells were evident as single cells, ranks, and clumps. Typical high columnar cells with normal nuclei were seen, but many cells were low in type, with nuclei characteristically irregular and fuzzy in contour and of variable size. Binucleate columnar cells were noted 4 times. Rare as is this observation it is of interest since, to our knowledge, nuclear fission has not been reported for surface epithelium. These few binucleate cells showed evidence of disorganization with extensive vacuolization of the cytoplasm. In every experiment but one, leucocytes and mucous neck chief cells were observed in the initial post-eugenol sample, as were a few parietal cells. The latter were well preserved with homogeneous cytoplasm and normal nuclear membranes.

An interesting observation in the early samples of this cycle is the presence of comparatively large organized clumps which had been exfoliated from the pouch wall. Some of these organized masses contained as many as eight hundred cells of variable morphology, and might be considered as tissue fragments (Fig. 12). They sometimes contained transitional cell types, ranging from small round or cuboidal cells to clongated columnar cells. Occasionally, the tissue fragment had mixed with it a few parietal and neck cells, as well as leucocytes. The cells were sometimes organized in palisade formation and included cells from the crypts as well as the surface of the mucosa. Metachromasia was consistently absent from the cytoplasm of these cells, but was present in the substratum of the smear.

In the latter part of the cycle (the last 2 half-hour samples), large numbers of neck chief cells and polys were noted, and the former were especially prominent. Thus, in the initial cycle following 30-36 hours for recovery, mucous neck chief cells were being removed to an extent not reached, comparatively, before the third cycle of the corresponding fatigue experiment. Many, if not most, of these neck chief cells showed no evidence, by staining, of intracellular mucin. In these last samples, occasional well preserved parietal cells were noted, but the relatively few columnar cells appeared abnormal. Their shapes varied, the cell membranes were hard to discern, and their nuclei were irregular in contour.

Cycle B (first follow-up): The eugenol wash had no features different from those of cycle A.

Throughout this cycle there could be noted metachromatically stained substances in the substrate, but no intracellular metachromasia was evident either in the columnar or in the neck chief cells. Several large tissue fragments were discerned in the post-eugenol smears. Occasional nicely preserved parietal cells were seen in all specimens of this cycle. A moderate number of neck chief cells and polys were evident in most of the samples. Comparatively few columnar cells were evident throughout this second cycle, and none in organized ranks. They were poorly preserved with cell walls frequently torn, and all intact thecae were non-metachromatic. The parietal cells were characteristically better preserved than the columnar or mucous neck cells.

THE SECOND FOLLOW-UP EXPERIMENTS

The gastric pouches were permitted to rest without further experimentation for 3–5 months, and then each was subjected to 3 or 4 cycles of stimulation and collection indentical with those described above. This number of cycles was established by criteria similar to those described for the first follow-up experiments.

Cycle A (second follow-up): Once again, the substratum of the smear prepared from the stimulating fluid contained metachromatic material. Moderate to large numbers of columnar cells were observed only within fragments macroscopically visible in the washings.

The first two half-hour specimens contained columnar cells in large quantities; many of these were typically normal high columnar cells (Fig. 11). The columnar cell clumps seen in these initial samples often consist of large aggregates of cells as in Fig. 12, which must not be mistaken for tissue sections. Thus, even after several months of recovery following the original fatigue experiments, the pouch mucosa seemed relatively friable and it was not difficult to dislodge epithelial cells in groups of several hundred. Some of the tissue fragments like those seen in Fig. 12 were covered with hemolyzed blood. In the substratum surrounding the cells, hemolyzed blood and metachromatic substances could be seen as patches of green and purple.

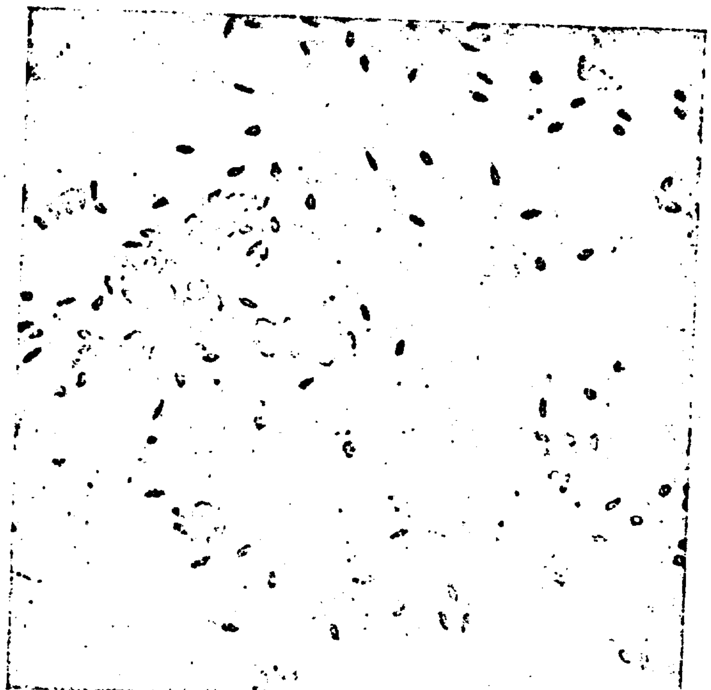

FIG. 11. Columnar cells observed in secretion obtained at the start of a second follow-up experiment (cycle A, sample 2). Well preserved, high columnar type of cell. 310×.

Mucous neck cells were evident as early as the second post-eugenol sample; they were always present, in every experiment, during the third half-hour period. Polys in moderate quantity appeared during the last two collection periods, characteristically enmeshed in fibrin masses, and suggestive of an inflammatory exudate (Fig. 13).

In the samples collected toward the end of this cycle, most of the cells were poorly preserved. The amount of greenish stained blood increased in successive samples throughout the cycle, and such blood sometimes even masked the intracellular metachromasia of neck chief cells. The latter were seen as single cells or, occasionally, in small ranks of 4–6 elements. Ranks of columnar cells, as already stated, usually contained much larger numbers than the smaller neck chief ranks.

Remaining cycles (second follow-up): The observations made in the cycles following the first tend to fall into a standard pattern. Macroscopically, blood clots were observed in the eugenol washings and these stained greenish in the smear preparations. The clots contained dense masses of cytolyzed cells, primarily columnar cells and their fragments.

The smears containing material from the succeeding samples appeared almost entirely green, indicative of the presence of hemolyzed blood. The latter was pervasive and tended to cover most cellular and other constituents in the secretions, making their study particularly difficult and hence unreliable. An occasional small patch of purplish metachromatic material was noted through the green. Thus, mucin may

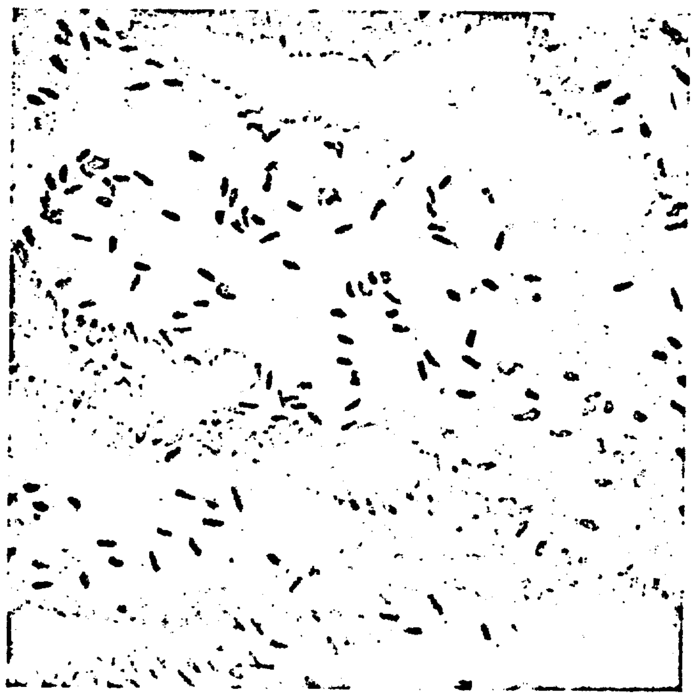

FIG. 12. Masses of columnar cells found in secretion obtained at the beginning of the second follow-up experiment (cycle A, sample 2). Large aggregates of columnar cells resemble small tissue fragments. 310×.

have been present more extensively than was suggested by the metachromasia, but it would have been masked by blood.

By the second and ensuing cycles, all cellular types excepting parietal cells—even neck chief cells and polys—appeared poorly preserved. In some of the samples, cytolysis was the characteristic feature, with cellular fragments alone being evident. Mucous neck cells and polys were present in moderate to large numbers and their aberrant appearance in these later cycles may be laid to extensive hemolysis of the erythrocytes.

COMPARATIVE RESPONSES OF THE DIFFERENT EXPERIMENTAL DOGS

The characteristics included in this study were arbitrarily graded on a scale of zero to four plus. To analyze graphically the interrelations among successive cycles, the intracyclic data for the four post-eugenol samples were averaged, and the means plotted as in Fig. 2. Each point thus represents the averaged data for any one cycle.

Some further points of information concerning this figure may well be given. Data for the fatigue experiments proper and the first and second follow-up experiments are presented for only 3 of the 4 animals. One dog (# 178) died shortly before the inauguration of the second follow-up experiment, and only the line graphs of the fatigue experiment and the initial follow-up are presented for this animal. Another (# 172) required but one cycle of stimulation and

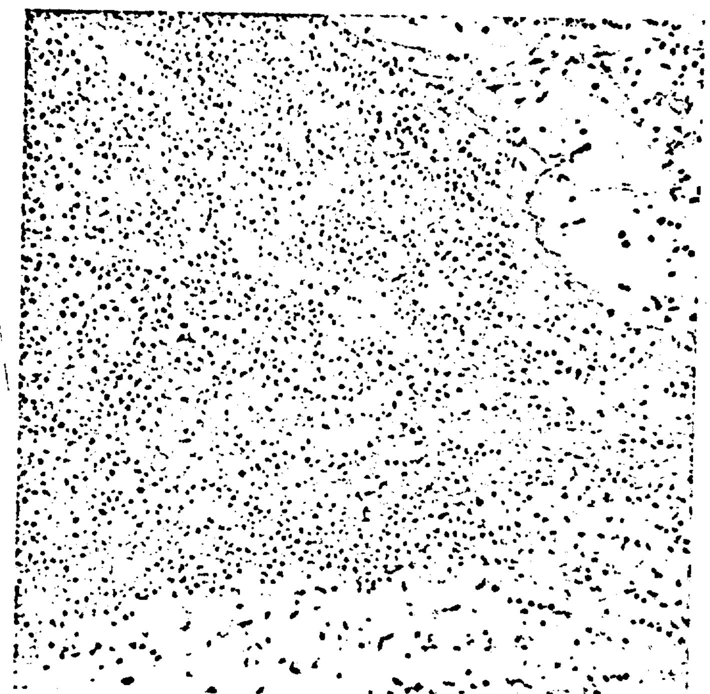

FIG. 13. Exudate containing polymorphonuclear leucocytes in a fibrin mesh observed during the second follow-up experiment (cycle A, sample 5). 70×.

collection in the first follow-up experiment, and not two cycles as with the other animals. The data for a fifth animal (the first to be studied) are not presented, since experiments on that dog were performed in order to develop procedures, and the concentration of eugenol was only 2%. However, results of this experiment are in no way in conflict with those for the other 4 dogs.

The line graphs indicate good uniformity in the responses of the several animals to successive stimuli. The fluctuations for any particular characteristic are relatively minor, whereas the trends show no striking disparity. The graphs, indicative of mucin and of single, clumped, and ranked columnar cells (the cells which are the chief producers of mucus in the corpus of the stomach), may be considered as a group because of their general similarity. On the other hand, those representative of the mucous neck chief cells and of the polys are also comparable with one another but dissimilar to the first mentioned group of graphs.

In the fatigue experiments as well as in those following recovery, the maximum of columnar cell exfoliation, whether as single cells or in groups, is in the initial cycle. This also holds true for the metachromatic material. Cellular and mucin contents of the smears diminish, and the last cycle of the fatigue experiment shows a value at or near zero. In the graphs of several of the fatigue experiments, a change of slope is noted after the third or fourth cycle; a comparable change in the curves for opacity and viscosity of these pouch secretions was reported by us elsewhere.[12] The incidence of organized ranks of columnar cells quickly diminishes, so that generally by the third cycle of the fatigue experiment, these are no longer evident.

Neck chief cells characteristically reach a peak during the second or third fatigue cycles. In only one instance (dog #172) is the maximum reached during the initial cycle, and maintained for a second cycle before diminishing. The line graphs for polys during the course of the fatigue experiments differ from all others in that they do not follow a characteristic pattern, and each graph has more than one change of slope. Peaks are reached at different times, twice in the second cycle, and once each during the third and sixth cycles. In all but one instance, polys are the only cellular constituents in the secretions which are present in greater numbers at the end of the fatigue experiments than during the first cycle of collections.

The graphs of the first follow-up experiments permit a few generalizations. Regarding the mucin and the three columnar cell graphs, it appears that in no instance is the index of the initial cycles as high as that of the initial cycles of the fatigue series. The response of the pouch mucosa at this time was more like that of the second or third cycle of the fatigue experiments. With respect to neck chief cells and polys, however, the initial cycles of the first follow-up series show a level near to or above the highest value attained throughout the course of the fatigue cycles. In 2 of the 4 animals, the first cycle was scored decidedly higher than in any of the fatigue cycles (#171 and #172). Having reached this level, the amounts of neck chief cells and polys either remain at this same high level during cycle B of the first follow-up or diminish slightly. As regards mucin and the columnar cell characteristics, the second cycle of this follow-up usually shows a marked diminution.

After 3 to 5 months of rest, the second follow-up experiments were performed. With the striking degree of uniformity, the graphs for mucin content and columnar cells (single, clumps and ranks) indicate that the levels of mucin and of cells in the first cycle do not reach those of the corresponding cycle of the fatigue series. They are, however, always higher than in the comparable cycle of the first follow-up experiments. Neck chief cells, on the other hand, are present in lesser numbers in the first cycle of the second follow-up than during any of the first follow-up cycles. In the ensuing cycles of the second follow-up, the chief cells show irregular fluctuations.

The amounts of polys present in the secretions show one interesting feature. The values of the initial cycles of the second follow-up and of the original fatigue series invariably demonstrate the presence of polys in approximately equal amounts. However, the second cycle of the second follow-up series has a score that is the same or higher than the maximum value attained during the earlier fatigue series—in two of three animals. By the third cycle, then, this level for polys is either maintained or elevated.

DISCUSSION

In recent years, investigations of workers in diverse fields have served to focus attention on the need for study of the cellular contents of various body fluids and secretions. Diagnostic smear techniques of different kinds have been elaborated, notably those of Papinicolaou and his colleagues, and studies have been made with the primary view of detecting cells in which a pathological transformation has already taken place. As is obvious to anyone attempting such studies, a cellular type may appear different when seen in a tissue section than when observed intact and unsectioned in a smear. Cells in smear preparation are not fragmented and are released from the pressures of neighboring cells. Long, tapering columnar cells, such as those pictured in Fig. 5, with an elongated post-nuclear portion are not typical of tissue sections. Columnar cells and granulocytes are not difficult to discern in smears, but the recognition of parietal cells and of mucous neck chief cells required considerable practice.

Body chief or peptic cells have never been observed with any surety in our smear preparations. There are several possibilities to account for this. Repeated stimulation with eugenol may never have resulted in desquamation of mucosa deep enough to release peptic cells from their *loci* in the glands; or, if released, the cells may have been too sensitive to withstand the manipulation involved in the preparations of smears; or, if present in the smears, they may have been missed by the observer because of their paucity. It appears to us that the first two possibilities are more probable than the last. In sections stained with toluidine blue, the zymogen granules of the peptic cells stain with virtually blue-black intensity. The basophilia of these cells has been commented upon by others. We feel, therefore, that had such cells appeared in our toluidine blue preparations, the basophilia would have served as a marker for the observer. At any rate, we have no observations on peptic cells to report in this study.

The parietal cells with their lightly staining cytoplasm (Fig. 7) have to be searched for carefully; they never appear in large numbers as do other types of cell, but they occur in many preparations. An interesting feature concerning parietal cells is that whenever noted they are well preserved. At stages of the experiments where other types of cells have undergone cytolysis, parietal cells are usually intact. Dislocation and subsequent treatment of these acid cells does not appear to harm them. Other evidence of their rugged quality is given by X-ray treatment which destroys other cellular components of the mucosa but leaves parietal cells morphologically intact.[1] Also, stomach tissue undergoing histolysis characteristically shows that parietal cells disintegrate later than do columnar or peptic cells.

The relative quantities of the various cells observed in the smears are roughly comparable with those present in tissue sections. Columnar cells are greatest by far in the smears and are desquamated earliest, as might be expected from their surface position. Mucous neck cells are next in quantity, reaching their peak later than do the surface cells. One notes small clusters of 3 to 7 neck cells but never high clumps or ranks as with columnar cells. Parietal cells appear in decidedly lesser numbers than do neck cells; peptic cells are simply not found.

The observations indicate that the effect of repeated stimulation with eugenol has resulted in a progressive exfoliation of parts of the mucosa. The extent of denudation can be ascertained only by similar experiments in which the eugenol stimulated pouch tissue is taken for histological examination. A series of such tissue specimens has been studied, and these permit some tentative comments.[8] The most interesting and significant is that one hour after removal of surface cells by the chemical stimulant, no denuded area is observed. The mucosal surface is covered by a layer of either flattened cuboidal, or tall columnar cells. When stained with toluidine blue, these new surfacing cells are usually seen to contain intracellular mucin. The time element cited in this instance is of some interest. Under normal physiological conditions, the gastric epithelial layer is subject to constant mechanical injuries and it is rather fortunate that processes exist whereby removed surface components are replaced by others which can quickly cover unprotected areas of underlying connective tissue. It must be emphasized that this type of experiment, with its gradual impairment of the gastric wall by means of a progressive "whittling" process, is different from the type of study conducted by Ferguson[2] wherein a small, deep area was removed—sometimes down to the muscularis—and the regenerative processes studied. The present work bears some resemblance to that reported by Grant,[3,4] but is more systematic and extensive. Our conclusions regarding the rate of replacement of the surface epithelial cells are in agreement with hers.

Are the effects of repeated eugenol stimulation comparable in any way to those occurring normally in the stomach? It is a fact, that under ordinary physiological conditions, epithelial cells are being shed continuously, and may be observed in gastric aspirates. The extent of this desquamatory activity is rarely, if ever, so exaggerated as that in Figs. 4, 5, 11, 12; even were it so, however, it is conceivable that the protective capacity of gastric epithelium is such as to cover quickly the underlying lamina propria. Hollander, Stein, and Lauber[10] report that desquamation of columnar cells may result even from mild treatment of dog's gastric mucosa with distilled water, NaCl in varied concentrations, and gentle massage. It is this type of observation which led Hollander[7] to propose that shedding of the glandular epithelium constitutes an additional protective device, together with the mucous secretion *per se.* Thus the concept of a "mucous barrier" has been extended to include both the secretion and the cells which prepare it. This compound barrier may explain the failure of attempts to induce, experimentally, malignant growths of the glandular portion of the stomach.[6, 7, 11] A comparison of Figs. 4 and 5 (from the beginning of the fatigue series) with Fig. 9 (from the end of the same series) indicates that repeated stimulation has damaged the barrier to the extent that well formed, normal appearing cells are no longer present in the secretion. Furthermore, in place of the opaque, viscous secretion of the first samples, the material collected at the end of the experiment is a decidedly less viscous, sero-sanguinous fluid, distinguished cellularly only by its leucocytes and fibrin mesh. If this concept of a defensive mucous barrier is valid, it is evident that after 5 or 6 cycles of repeated stimulation with eugenol emulsion, the protective device of the gastric wall has been extensively impaired, and that the mucosa in such a condition may, if subjected to a suitable agent, undergo malignant transformation.

SUMMARY

Repeated stimulation of Heidenhain pouches of 5 dogs has been performed with a eugenol emulsion. After set periods of rest, two follow-up experiments consisting of similar cycles of stimulation and collection were inaugurated. Toluidine blue-stained smears of all specimens were prepared; their study indicates that:

(1) Eugenol effectively and progressively desquamates many components of the gastric mucosal wall. Columnar cells, parietal cells, and mucous neck chief cells may be recognized in the smears. Only peptic (body chief) cells were not evident in the preparations, and reasons for their absence are presented.

(2) Columnar cells in great density are found in the earliest samples; their number diminishes with repeated stimulation. This drop parallels a diminution in metachromatically stained mucin-like substances.

(3) Mucous neck chief cells, with intra-cytoplasmic metachromasia, appear initially in the secretions later than columnar cells, as do polymorphonuclear leucocytes.

(4) Parietal cells can be observed in most samples, but in lesser quantity than any other cellular entity. When other cells appear poorly preserved, or even cytolyzed, the parietal cells appear normal.

(5) Blood, staining greenish, increases with additional stimulation.

(6) After either 6 or 7 repeated cycles of stimulation and collection, the specimens contain few or no columnar or neck chief cells and little mucinous material. They consist, typically, of inflammatory exudate with blood, fibrin and polymorphonuclear leucocytes. The mucous barrier which protects the underlying glandular epithelium, discussed in this report, has been impaired.

(7) The protective barrier has not been permanently injured but can recover, as evidenced by results of the follow-up experiments. Recovery of the mucosa to its original state, however, is not complete, as is shown by early bleeding and by the frequent dislodgement of fragments of mucosal wall, occasionally comprising several hundreds of cells.

(8) Comparative responses of 4 of the experimental animals are described and comparative charts are presented. Photomicrographs illustrating certain features of the experiments are interpolated throughout the paper.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 6, 378-387 (1964)

A Comparison of the Toxicity of Some Allyl, Propenyl, and Propyl Compounds in the Rat[1]

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Many aliphatic allyl and propyl esters and many aromatic compounds containing the allyl, propenyl, or propyl groups are used as flavoring agents in foods and beverages. In the work described here, the toxicity of a number of compounds containing these 3-carbon groups has been compared to determine whether toxicity can be related to structure. The propyl group is a saturated, straight-chain, 3-carbon group. The allyl group ($CH_2{=}CHCH_2{-}$) differs only in the presence of a double bond. The propenyl group ($CH_3CH{=}CH{-}$) is isomeric to the allyl group and differs only in the location of the double bond. The criteria used in the comparison of toxicity were the oral LD_{50}'s and the occurrence of macroscopic liver lesions in a short-term, high-dose study. The aliphatic compounds tested were allyl alcohol, five allyl esters, propanol, and three propyl esters. The aromatic compounds tested included allyl, propyl, and propenyl analogs of four types of benzene derivatives. The aromatic compounds containing only the substituents other than the 3-carbon group or containing the aldehyde group in the same position as the 3-carbon side chain were also tested.

Hepatotoxicity was investigated because of the known effects of some allyl compounds on the liver. Single doses of allyl alcohol and allyl formate produce peripheral necrosis of the liver (Eger, 1956; Popper and Schaffner, 1957). Repeated doses of allyl formate can cause cirrhosis (Krüskemper and Hartmann, 1955; Popper and Schaffner, 1957). In contrast, aliphatic propyl compounds have not been associated with liver damage. Safrole, an aromatic compound containing the allyl group, has been shown to be hepatotoxic. It is a weak hepatic carcinogen when fed chronically in the diet of rats (Long *et al.*, 1963). Dihydrosafrole and isosafrole (the propyl and propenyl analogs of safrole) have also been found to be hepatotoxic when fed chronically in the diet to rats (Hagan *et al.*, 1964).

METHODS AND MATERIALS

Acute Toxicity

The acute oral toxicity of each compound was determined in rats of the Osborne-Mendel or Sherman strain. Young, adult rats in the weight range 180–350 g were used. The compounds were administered by stomach tube to rats fasted 18 hours. A group of five males and five females was used at each dose level. The rats were observed until the survivors had returned to normal in appearance and weight. LD_{50}'s were computed by the method of Litchfield and Wilcoxon (1949).

[1] Presented in part at the American Industrial Hygiene Conference, May 6–10, 1963, at Cincinnati, Ohio.

All the compounds except piperonal and vanillin were liquids. They were administered undiluted except for the following (solution percentages are w/v): allyl alcohol (2% in water), allyl formate (5% in corn oil), allyl acetate (10% in corn oil), allyl heptylate (50% in corn oil). Piperonal was administered as a 25% solution in corn oil. The solution was maintained at 37–40°C during use to prevent precipitation of the solute. Vanillin was administered as a 20% solution in propylene glycol.

Macroscopic Liver Lesions

The capacity of each compound to produce macroscopic liver lesions was determined in groups of three male and three female rats by using a dose of approximately one-third the LD_{50} of the compound. This dose was administered daily by stomach tube for 4 days. The rats were sacrificed on the fifth day, and the livers were examined for gross lesions. The following criteria were used for rating the severity of the lesions seen with the aliphatic compounds: *0*, normal; *1*, slightly grayish or yellowish in color, mottling, presence of one or two small yellow or yellowish-white necrotic areas in one or two lobes (livers with normal color and the same number of necrotic areas were also rated *1*): *2*, normal or yellowish in color with small necrotic areas in all lobes; *3*, yellow in color with approximately half the liver necrotic: *4*, extreme yellow discoloration with most or all of the liver necrotic and very brittle in texture. A different set of criteria was used for the rating of the lesions produced by the aromatic compounds: *0*, normal: *1*, slight discoloration (yellowish or grayish), slight mottling, blunt appearance to the edges of the lobes without enlargement; *2*, moderate yellow discoloration with or without mottling, fatty appearance, small white streaks, slight enlargement; *3*, marked yellow discoloration with or without mottling, fatty appearance, small white streaks, soft and mushy texture, moderate enlargement. The ratings for the individual livers were averaged to give a rating for each compound.

Source of Materials

The compounds used were obtained from the following sources: allyl alcohol, *n*-propanol—Fisher Scientific Co.; benzene—Merck and Co., Inc.; eugenol—Magnus, Mabee, and Reynard, Inc.; allyl butyrate—Ungerer and Co.; allyl acetate, allyl formate, anisole, isosafrole, *n*-propyl *n*-butyrate, *n*-propyl formate, *n*-propyl acetate—Eastman Organic Chemicals; allyl caproate, allyl heptylate, anethole, anisaldehyde, benzaldehyde, dihydroanethole, dihydrosafrole, isoeugenol, piperonal, safrole, vanillin—Dodge and Olcott; allylbenzene, estragole, guaiacol, methylenedioxybenzene, propenylbenzene, propylbenzene—K & K Laboratories, Inc. Methylenedioxybenzene was also synthesized in our laboratory by the method of Shorygin *et al.* (1938).

RESULTS AND DISCUSSION

Acute Toxicity

The LD_{50}, slope function, toxic signs, and death time for each compound are listed in Table 1. In the table, the aliphatic compounds have been divided into two groups, one containing the allyl compounds and the other the propyl compounds. The aromatic compounds have been grouped according to the substituents on the benzene ring other than the 3-carbon side chains or aldehyde group.

The greatest difference in acute toxicity among the allyl, propenyl, and propyl com-

TABLE 1
ACUTE ORAL TOXICITY OF SOME ALLYL, PROPENYL, PROPYL, AND RELATED COMPOUNDS IN RATS

Compound	LD_{50} (19/20 confidence limits) (mg/kg)	Slope function (19/20 confidence limits)	Toxic signs	Death time
Aliphatic allyl compounds				
Allyl alcohol	70 (63–79)	1.6 (1.2–2.0)	Depression, colorless secretion from eyes, diarrhea, scrawny appearance for several days	4 hours–4 days
Allyl formate	124 (107–144)	1.3 (0.5–3.6)	Depression, scrawny appearance for several days	4 hours–5 days
Allyl acetate	142 (116–175)	1.6 (1.3–2.0)	Depression, rough fur, and scrawny appearance for several days	4 hours–6 days
Allyl butyrate	250 (216–290)	1.5 (1.2–1.8)	Depression, wet posterior, scrawny appearance for several days	4 hours–5 days
Allyl caproate	218 (186–255)	1.3 (1.1–1.5)	Depression, scrawny appearance	4–18 hours
Allyl heptylate	500 (392–638)	1.7 (1.2–2.2)	Depression, ataxia	2–18 hours
Aliphatic propyl compounds				
n-Propanol	6500 (5800–7280)	1.2 (1.1–1.3)	Comatose within a few minutes after treatment, weight loss on high doses, scrawny appearance	2–18 hours
Propyl formate	3980 (3350–4740)	1.5 (1.2–1.7)	Depression, normal in appearance day after treatment	4–18 hours
Propyl acetate	9370 (7670–11,430)	1.5 (1.1–1.8)	Depression, rough fur, and scrawny appearance for several days	4–18 hours
Propyl butyrate	15,000 (12,605–17,850)	1.4 (0.8–2.5)	Depression, some rats comatose for several hours, rough fur and diarrhea	1–3 days
Benzene derivatives				
Benzene	4080 (3260–5100)	1.7 (1.4–2.1)	Tremors, ataxic and comatose on high doses, scrawny appearance for several days	1 hour–4 days

TABLE 1 (*Continued*)

	LD_{50}	Slope

n-Propanol	6500 (5800–7280)	1.2 ([illegible]–1.3)	Comatose within a few minutes after treatment, weight loss on high doses, scrawny appearance	2–18 h[illegible]
Propyl formate	3980 (3350–4740)	[illegible].5 (1.2–1.7)	Depression, normal in appearance day after treatment	4–18 hours
Propyl acetate	9370 (7670–11,430)	1.5 (1.1–1.8)	Depression, rough fur, and scrawny appearance for several days	4–18 hours
Propyl butyrate	15,000 (12,605–17,850)	1.4 (0.8–2.5)	Depression, some rats comatose for several hours, rough fur and diarrhea	1–3 days
Benzene derivatives				
Benzene	4080 (3260–5100)	1.7 (1.4–2.1)	Tremors, ataxic and comatose on high doses, scrawny appearance for several days	1 hour–4 days

TABLE 1 (*Continued*)

Compound	LD_{50} (19/20 confidence limits) (mg/kg)	Slope function (19/20 confidence limits)	Toxic signs	Death time
Allylbenzene	5540 (4620–6650)	1.5 (1.2–2.0)	Depression, some rats comatose for 2–3 days, wet posterior, scrawny appearance for several days	4 hours–5 days
Propenylbenzene	3600 (2650–4900)	2.2 (1.3–3.8)	Scrawny appearance, weight loss, and wet posterior for 5–7 days after treatment	4 hours–9 days
Propylbenzene	6040 (4830–7550)	1.5 (1.2–2.0)	Depression, rough fur, and scrawny appearance	1–3 days
Benzaldehyde	1300 (1110–1540)	1.4 (1.2–1.6)	Depression, comatose on higher doses	4–18 hours
Methoxybenzene derivatives				
Anisole (methoxybenzene)	3700 (3240–4220)	1.2 (1.1–1.4)	Depression, porphyrinlike deposit around eyes, salivation, bloody urine, rough fur	4 hours–8 days
Estragole (1-methoxy-4-allylbenzene)	1820 (1670–1980)	1.2 (1.1–1.2)	Marked depression, some rats comatose for 24 hours, rough fur, wet posterior, porphyrinlike deposit around eyes	4 hours–8 days
Anethole (1-methoxy-4-propenylbenzene)	2090 (1420–3070)	1.8 (1.3–2.4)	Depression with low doses, comatose with higher doses	4 hours–4 days
Dihydroanethole (1-methoxy-4-propylbenzene)	4400 (3380–5720)	1.9 (0.6–5.6)	Depression, porphyrinlike deposit around eyes and nose, wet posterior	4 hours–3 days
Anisaldehyde (*p*-methoxybenzaldehyde)	1510 (1360–1700)	1.2 (1.1–1.3)	Depression	4–18 hours
Methylenedioxybenzene derivatives				
Methylenedioxybenzene	580 (487–690)	1.4 1.2–1.7)	Depression, comatose on high doses, porphyrinlike deposit around eyes, rough fur	4 hours–6 days
Safrole (1,2-methylenedioxy-4-allylbenzene)	1950 (1760–2160)	1.3 (0.6–3.0)	Depression, ataxia, diarrhea	4 hours–5 days

TABLE 1 (*Continued*)

Compound	LD_{50} (19/20 confidence limits) (mg/kg)	Slope function (19/20 confidence limits)	Toxic signs	Death time
Isosafrole (1,2-methylenedioxy-4-propenylbenzene)	1340 (1140–1590)	1.4 (1.2–1.7)	Depression, coma, rough fur, loss of equilibrium, porphyrinlike deposit around eyes and nose, scrawny appearance	4 hours–8 days
Dihydrosafrole (1,2-methylenedioxy-4-propylbenzene)	2260 (1840–2780)	1.7 (1.4–2.0)	Depression, colorless secretion from eyes, scrawny appearance	2–5 days
Piperonal (3,4-methylenedioxybenzaldehyde)	2700 (2350–3100)	1.5 (1.1–2.0)	Excitable for several hours, then depression and ataxia	2 hours–5 days
Hydroxymethoxybenzene derivatives				
Guaiacol (1-hydroxy-2-methoxybenzene)	725 (302–1740)	3.2 (0.3–30)	Marked depression, comatose on higher doses	1 hour–4 days
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	2680 (2420–2970)	1.2 (1.1–1.4)	Comatose soon after treatment	0–2 hours
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)	1560 (1290–1880)	1.4 (1.4–1.5)	Comatose, scrawny appearance for several days	1 hour–7 days
Vanillin (3-methoxy-4-hydroxybenzaldehyde)	1580 (1390–1810)	1.3 (1.2–1.5)	Comatose soon after treatment, scrawny appearance for several days	4 hours–4 days

pounds tested was seen in the aliphatic allyl and aliphatic propyl compounds. Allyl alcohol and the allyl esters were much more acutely toxic than propanol and the propyl esters. The LD_{50}'s for allyl alcohol, allyl formate, allyl acetate, and allyl butyrate ranged from 70 to 250 mg per kilogram of body weight while the LD_{50} range for the corresponding propyl compounds was 3980 to 15,000 mg/kg. Allyl caproate and allyl heptylate had LD_{50}'s of 218 and 500 mg/kg. The corresponding propyl compounds were not tested. As the acid portion of the ester increased in chain length, the toxicity decreased in both the allyl and propyl series. Allyl caproate was an exception to this general trend. Its LD_{50} was slightly less than that of allyl butyrate, 218 vs. 250 mg/kg.

For some, but not all, of the allyl compounds, the LD_{50} could be related to the allyl portion of the molecule. The LD_{50}'s of allyl alcohol, allyl formate, allyl acetate, and allyl caproate contained nearly equal amounts by weight of the allyl group: 50 mg in the LD_{50} of allyl alcohol, 70 mg/kg; 59 mg in the LD_{50} of allyl formate, 124 mg/kg; 58 mg in the LD_{50} of allyl caproate, 218 mg/kg. The LD_{50}'s of allyl butyrate and allyl heptylate contained larger amounts by weight of the allyl group: 80 mg in an LD_{50} of 250 mg/kg and 120 mg in an LD_{50} of 500 mg/kg, respectively.

The allyl and propyl groups differ only in the presence of the double bond in the allyl group; the difference in toxicity between the two types of aliphatic compounds must be due to this unsaturation.

The effect of the 3-carbon groups on toxicity, when they were substituents on the benzene ring, was seen with allylbenzene, propenylbenzene, and propylbenzene. The difference in acute toxicity of the aromatic allyl and propyl compounds was much less than the difference between the aliphatic allyl and propyl compounds. The LD_{50} of allylbenzene was 5540 mg/kg vs. an LD_{50} of 6040 mg/kg for propylbenzene. Both compounds were less toxic than benzene ($LD_{50} = 4080$ mg/kg). The substitution of the propenyl group for the isomeric allyl group resulted in a compound that was more toxic ($LD_{50} = 3600$ mg/kg) than the allyl analog and also probably slightly more toxic than benzene. Allylbenzene is readily convertible to propenylbenzene *in vitro*, and Williams (1959) has suggested that this reaction also takes place *in vivo*. However, the difference in acute toxicity of the two compounds suggests that the conversion reaction, if it does take place, is very slow or is incomplete.

When the benzene ring contained additional substituents, i.e., the methoxy group, the methylenedioxy group, or the hydroxy and methoxy groups ortho to each other, the acute toxicities of the resulting compounds were greater than that of benzene whether or not the compound contained one of the 3-carbon side chains. The propyl derivatives, dihydroanethole and dihydrosafrole, were less toxic than the allyl derivatives, estragole and safrole; but, like allylbenzene and propylbenzene, these aromatic compounds did not show the marked difference in toxicity seen with the aliphatic allyl and propyl compounds. The propenyl compounds, isosafrole and isoeugenol, were more toxic than their allyl analogs, safrole and eugenol; but anethole was less toxic than its allyl analog, estragole. Methylenedioxybenzene ($LD_{50} = 580$ mg/kg) and guaiacol ($LD_{50} = 725$ mg/kg), which contained neither a 3-carbon side chain nor the aldehyde group, were the most toxic of the aromatic compounds tested.

The aromatic aldehydes were tested to determine the effect of a substituent, other than the 3-carbon side chains, that occupied the same position on the benzene ring.

However, the aldehydes showed no consistent relationship in their toxicity to the other derivatives of their groups. Benzaldehyde and anisaldehyde were the most toxic compounds in their groups, piperonal was the least toxic of its group, and vanillin was similar in toxicity to isoeugenol in its group.

TABLE 2

MACROSCOPIC LIVER LESIONS IN RATS FROM REPEATED DOSES OF SOME ALLYL, PROPENYL, PROPYL, AND RELATED COMPOUNDS

Compound	Dose[a] (mg/kg)	Mortality ratio[b]	Macroscopic liver lesions, average rating[c]
Aliphatic allyl compounds			
Allyl alcohol	25	0/12[d]	1.5
Allyl formate	40	0/6	2
Allyl acetate	45	0/6	2.5
Allyl butyrate	85	0/6	1.5
Allyl caproate	75	0/6	2.5
Allyl heptylate	165	1/6	3
Aliphatic propyl compounds			
n-Propanol	2160	0/6	0
Propyl formate	1330	0/6	0
Propyl acetate	3120	0/6	0
Propyl butyrate	5000	0/6	0
Benzene derivatives			
Benzene	1360	1/6	0
Allylbenzene	1850	1/6	0
Propenylbenzene	1200	2/6	1.5
Benzaldehyde	435	1/6	0
Methoxybenzene derivatives			
Anisole	1230	1/6	0
Estragole	605	0/6	0.5
Anethole	695	1/6	0.5[e]
Dihydroanethole	1470	0/6	0.5
Anisaldehyde	500	0/6	0.5
Methylenedioxybenzene derivatives			
Methylenedioxybenzene	190	0/6	0.5
Safrole	650	0/6	2.5
Isosafrole	460	2/6	2.5
Dihydrosafrole	770	1/6	2.5
Piperonal	900	0/6	0.5
Hydroxymethoxybenzene derivatives			
Guaiacol	240	2/6	0.5
Eugenol	895	0/6	1
Isoeugenol	520	1/6	0
Vanillin	530	0/6	0

[a] Dose = approximately 1/3 LD_{50}.
[b] Mortality ratio = number of rats dying during test period per total number of rats.
[c] See text for criteria used in rating the macroscopic liver lesions.
[d] Two groups of six rats were treated at different times.
[e] The liver from one rat in this group had small necrotic areas like those seen in the livers of rats treated with aliphatic allyl compounds.

p in their toxicity to the other dehy' were the most toxic of it group, and vanillin was

SOME ALLYL, PROPENYL, PROPYL,

Mortality ratio[b]	Macroscopic liver lesions, average rating[c]
0/12[d]	1.5
0/6	2
0/6	2.5
0/6	1.5
0/6	2.5
1/6	3
0/6	0
0/6	0
0/6	0
0/6	0
1/6	0
[illegible]	0
[illegible]	1.5
1/6	0
1/6	0
0/6	0.5
1/6	0.5[e]
0/6	0.5
0/6	0.5
0/6	0.5
0/6	2.5
2/6	2.5
1/6	2.5
0/6	0.5
2/6	0.5
0/6	1
1/6	0
0/6	0

otal number of rats.

ke those seen in the livers of

Despite the variation in structures represented by the compounds tested, all the compounds except piperonal were depressants. Piperonal caused prolonged excitation followed by depression. Deaths were delayed with allyl alcohol, the shorter chain allyl esters, propyl butyrate, and most of the aromatic compounds. Allyl caproate, allyl heptylate, most of the aliphatic propyl compounds, eugenol, anisaldehyde, and benzaldehyde had death times of less than 24 hours.

Macroscopic Liver Lesions

The average ratings assigned to the macroscopic liver lesions seen after four daily doses of the test compounds are listed in Table 2 with the dose used and the mortality ratio for each compound. The doses used (1/3 LD_{50}'s) were related to the acute toxicity. Since the acute toxicities of these compounds are not due solely to liver damage, the doses would not be expected to be equally hepatotoxic.

All the aliphatic allyl compounds produced gross liver lesions; none of the aliphatic propyl compounds did. The lesions produced by the aliphatic allyl compounds consisted of discoloration ranging from slight (gray or yellow hue) to severe ("massive yellow atrophy"), mottling ranging from slight to marked, and necrosis. The necrotic areas were yellow or yellowish-white in color, irregular in shape, and, generally, slightly raised above the surface. The areas ranged in size from small spots (1–2 mm in diameter) to complete involvement of a lobe. The caudate lobe and posterior lobule of the right lateral lobe were most frequently involved. The small necrotic areas were occasionally seen in livers that looked normal otherwise.

Considerable variation in the amount of liver damage was seen in the individual animals receiving allyl alcohol, allyl formate, and allyl butyrate. The livers from some animals in these groups appeared normal; only one or two animals from each of these groups showed liver damage severe enough to be rated *3* or *4*. In contrast, none of the livers from the rats receiving allyl acetate, allyl caproate, and allyl heptylate was rated as normal. For allyl acetate and allyl caproate, most of the livers were rated *2* or *3*. Allyl heptylate appeared to be the most hepatotoxic of the compounds tested under these conditions. The liver lesions in three of the five surviving animals were severe enough to be rated *4*. With all the aliphatic compounds, the general condition of the animals remained good during the test period. Weight loss was minimal even in rats with severely damaged livers, and there was only one death in the rats treated with the aliphatic compounds.

The hepatotoxicity seen with the aromatic compounds, in contrast to that seen with the aliphatic compounds, could not be attributed to a specific 3-carbon side chain. Benzene, allylbenzene, benzaldehyde, anisole, isoeugenol, and vanillin were not hepatotoxic under the conditions of this test. The absence of hepatotoxicity with allylbenzene indicates that attaching the allyl group to the benzene ring appears to eliminate its hepatotoxicity as well as reduce its acute toxicity.

Mild liver lesions, consisting of slight discoloration, mottling, and blunting of the lobe edges, were seen with a number of the aromatic compounds. There was no consistent relationship to structure in these findings. The lesions were found in compounds with no 3-carbon side chain (guaiacol, methylenedioxybenzene), the allyl side chain (estragole, eugenol), the propenyl side chain (anethole), the propyl side chain (dihydroanethole), and the aldehyde group (anisaldehyde, piperonal). Propenylbenzene, which differs from allylbenzene only in the location of the double bond in the 3-carbon side chain, caused moderate liver damage consisting of discoloration, mottling, blunting of the lobe edges, and slight enlargement.

Severe liver lesions in the case of the aromatic compounds were found only with safrole, isosafrole, and dihydrosafrole. These compounds caused discoloration, a fatty appearance, loss of normal texture, and enlargement. The yellow discoloration was never as marked as that seen with the most severely damaged livers from the aliphatic allyl compounds. Infrequently, small, white streaks, usually indented below the surface of the liver, were seen. The enlargement and loss of normal texture appeared to involve all the lobes equally. Mottling was seen in some of the livers but not in all. All the livers from the rats treated with safrole, isosafrole, and dihydrosafrole were rated *2* or *3*, with the exception of a liver from a safrole-treated rat which was rated *1*. Since such hepatic lesions were found only in the three compounds having both the methylenedioxy substituent and one of the 3-carbon side chains and not in methylenedioxybenzene or in piperonal, both substituents must be necessary for the hepatotoxicity of the three compounds. Hagan *et al.* (1964) and Long and Jenner (1963) have shown that in subacute and chronic studies the three compounds have a similar qualitative effect on the liver but differ in the degree of damage to the liver and in their effect on other tissues.

Many of the rats treated with the aromatic compounds lost weight, especially in the anethole, safrole, isosafrole, dihydrosafrole, guaiacol, eugenol, and isoeugenol groups, and were in poor condition at the end of the test. There were one or two deaths in many of the groups.

The finding of liver damage in a test such as that described here does not indicate whether or not the compound will be hepatotoxic in longer studies. Feeding studies in rats have been carried out in our laboratory on several of the compounds examined in the work described here. Allyl caproate did not cause liver damage when fed at a level of 0.19% in the diet of rats for 1 year, nor did benzaldehyde, anisaldehyde, eugenol, isoeugenol, or piperonal fed at a level of 1% for 15–19 weeks. Anethole caused slight hydropic changes in the hepatic cells (males only) when fed at a level of 1% in the diet to rats for 15 weeks, but no hepatic damage was seen on a level of 0.25% for 1 year. Allyl heptylate caused degeneration of hepatic cells and formation of new bile ducts at dietary levels of 0.1 to 1% for 17 weeks. A chronic feeding study on this compound is currently in progress.

SUMMARY

The toxicity in the rat of a number of allyl, propenyl, propyl, and related compounds has been compared using as criteria the acute oral toxicity and the hepatotoxicity in a short-term, high-dose study. The results of these studies indicated:

1. Aliphatic allyl compounds (allyl alcohol and allyl esters) were much more acutely toxic than aliphatic propyl compounds (propanol and propyl esters).

2. The aliphatic allyl compounds caused macroscopic liver lesions, the most significant of which was necrosis; the propyl compounds were not hepatotoxic.

3. Aromatic allyl compounds were more toxic than aromatic propyl compounds, but the difference was not as marked as with the aliphatic allyl and propyl compounds. Aromatic propenyl compounds were usually, but not always, more toxic than aromatic allyl compounds.

4. The nature of other substituents on the benzene ring influenced the hepatotoxicity of the aromatic allyl, propenyl, and propyl compounds. Allylbenzene was not hepatotoxic. Safrole, isosafrole, and dihydrosafrole—the allyl, propenyl, and propyl derivatives of methylenedioxybenzene—

caused severe liver lesions consisting of discoloration, enlargement, and loss of normal texture. The hepatotoxicity of these compounds appeared to be independent of the nature of the 3-carbon side chain, but both the 3-carbon side chain and the methylenedioxy group appeared to be necessary for the hepatotoxic action. Mild liver lesions, discoloration, mottling, and blunting of the lobe edges were seen with a number of the other aromatic compounds tested, but the occurrence of these lesions was not related to a particular structure.

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ammonium phosphate urinary calculi in either sterile or infected urine. Acidification of the urine is usually impossible in the presence of infection with urea-splitting bacteria and acidification decreases the concentration of citric acid in the urine. Shore also advised a low phosphorus diet and aluminum hydroxide orally; this decreases excretion of phosphate in the urine to as little as 10 per cent of its previous level but does not disturb the metabolic balance of calcium, phosphorus or nitrogen in these patients.

Some success (see Hamer and Mertz, *J. Urol.*, 1944, **52**, 475) has been obtained in dissolving stones already formed in the bladder or even the renal pelvis by continuous irrigation with *solution "G"*, which consists of citric acid monohydrate 32.25 Gm., magnesium oxide (anhydrous) 3.84 Gm., sodium carbonate 4.37 Gm. and sterile distilled water to make 1000 ml. Continuous irrigation, or repeated instillation, with 1.5 to 3 liters of this solution daily is required for 10 days or much longer to dissolve large stones. *Solution "M"* differs from *"G"* in using sodium carbonate 8.84 Gm.; it is less irritating but likewise less acid and less solvent. Citric acid will not dissolve calcium oxalate, uric acid or cystine stones.

Rickets.—The daily addition to the feeding formula of the infant of 30 ml. of molar sodium citrate (294 Gm. of $Na_3C_6H_5O_7.2H_2O$ per liter) and 20 ml. of molar citric acid (210 Gm. of $H_3C_6H_5O_7.H_2O$ per liter) cures rickets in the infant at about the same rate as with large doses of vitamin D (Hamilton and Dewar, *Am. J. Dis. Child.*, 1937, **54**, 548; Shohl, *J. Nutrition*, 1937, **14**, 69). In active rickets, the blood citrate concentration is low and is elevated rapidly when vitamin D is administered. Citrate therapy, however, will not prevent rickets; an adequate diet including vitamin D is essential.

Miscellaneous Uses.—Citric acid is mildly astringent and is sometimes used in inflamed conditions of the skin, such as sunburn. A concentration of about 1.6 per cent has been used in collyria, and up to 20 per cent has been employed in mouth washes.

Citric acid, in the form of citric acid syrup, is used as a vehicle for salty drugs. The acid is sometimes employed in formulations containing iron salts and tannin to retard development of color. The acid is also an important ingredient of effervescent salts, the water of hydration present in it serving to provide sufficient water to produce the pasty mass essential for the formation of a granular salt. Citric acid is included in several U.S.P. and N.F. preparations.

Dose.—The usual dose of the acid is 0.3 to 2 Gm. (approximately 5 to 30 grains).

Storage.—Preserve "in tight containers." *U.S.P.*

Labeling.—"Label Citric Acid to indicate whether it is anhydrous or hydrous. Where the quantity of Citric Acid is indicated in the labeling of any preparation containing Citric Acid, such indication is in terms of anhydrous Citric Acid, unless otherwise specified in the monograph." *U.S.P.*

CITRIC ACID SYRUP. U.S.P. (B.P.)

[Syrupus Acidi Citrici]

B.P. Syrup of Lemon; Syrupus Limonis. Sirupus Citri. *Fr.* Sirop d'acide citrique; Sirop de lemon. *Ger.* Kunstlicher Citronensirup. *It.* Sciroppo di limone. *Sp.* Jarabe de limón; *Jarabe de Acido Citrico.*

Dissolve 10 Gm. of citric acid in 10 ml. of purified water, add 950 ml. of syrup and mix well. Add 10 ml. of lemon tincture, and enough syrup to make 1000 ml. Mix thoroughly. *This preparation must not be dispensed if it has a terbinthinate odor or taste or shows other indications of deterioration. U.S.P.*

The B.P. calls this preparation Syrup of Lemon, and makes it by macerating fresh lemon peel in a small quantity of alcohol to which is added, after filtering, citric acid and syrup. The proportion of citric acid in the finished product is 2.4 per cent w/v. The B.P. directs that it should be stored in a container which has previously been washed with boiling water and kept in a cool place. The acidity of the B.P. product is more than twice that of the U.S.P. preparation.

Alcohol Content.—Less than 1 per cent, by volume, of C_2H_5OH. *U.S.P.*

Use.—Because of its acidity, as well as sweetness, this syrup has utility as a vehicle for bitter or salty drugs; it has no remedial value. Its acid reaction may give rise to incompatibilities with alkaline ingredients, such as phenobarbital sodium, from which it precipitates phenobarbital.

Storage.—Preserve "in tight containers, preferably at a temperature not above 25°." *U.S.P.*

CLOVE. N.F., B.P.

Caryophyllus, Cloves

"Clove is the dried flower-bud of *Eugenia caryophyllata* Thunberg (Fam. *Myrtaceæ*). Clove yields, from each 100 Gm., not less than 16 ml. of clove oil." *N.F.* The B.P. recognizes *Eugenia caryophyllus* (Spreng.) Sprague as the botanical origin of Clove. This is merely a botanical synonym for *Eugenia caryophyllata* Thunb.

Caryophyllum; Caryophylli Aromatici; Flores Caryophylli; Clavi Aromatici. *Fr.* Girofle; Clou de girofle. *Ger.* Gewürznelken; Kneidenelken; Nägelein. *It.* Chiodi di garofano. *Sp.* Clavo de especia; *Clavo. Portug.* Clavo da India. *Dutch.* Kruidnagel.

The clove-tree is a small tree inhabiting the Molucca Islands and southern Philippines. It has a pyramidal form, is evergreen, and is adorned throughout the year with a succession of beautiful rosy flowers. The stem is of hard wood, and covered with a smooth, grayish bark. The leaves are opposite, petiolate, about four inches in length by two in breadth, obovate-oblong, acuminate at both ends, entire, sinuate, with many parallel veins on each side of the midrib. They have a firm consistence and a shining green color, and when bruised are highly fragrant. The flowers are disposed in terminal corymbose panicles, and exhale a strong, penetrating, and grateful odor.

The natural geographical range of the clove is extremely limited, being confined to the Molucca or, as they were one time called, Clove Islands. According to Flückiger, cloves were known in western Europe as early as the sixth century, long before the discovery of the Moluccas by the Portuguese. After the conquest of the Molucca Islands by the Dutch, the monopolizing policy of that commercial people led them to extirpate the trees in nearly all the islands except Amboyna and Ternate, which were under their immediate inspection. Notwithstanding their vigilance, a French governor of the Islands of France and Bourbon, named Poivre, succeeded, in 1770, in obtaining plants from the Moluccas and introducing them into the colonies under his control. Five years afterward the clove-tree was introduced into Cayenne and the West Indies, in 1803 into Sumatra, and in 1818 into Zanzibar. At present the spice is cultivated both in the West and East Indies, in tropical Africa, and in Brazil. Approximately three-fourths of the world's clove supply is grown in Zanzibar and the neighboring island of Pemba.

The unexpanded flower buds are the part of the plant employed under the ordinary name of cloves. They are first gathered when the tree is about six years old. The fruit has similar aromatic properties, but much weaker. The buds are at first white, then become green, and then bright red. They are gathered when their lower part turns from green to red. This is done by hand picking from movable platforms or by beating the trees with bamboos and catching the falling buds. In the Moluccas they are said to be sometimes immersed in boiling water and afterward exposed to smoke and artificial heat before being spread out in the sun. In Zanzibar, Pemba, Cayenne, and the West Indies they are dried simply by solar heat, often on mats, and separated from their pedicels and peduncles ("clove stems"). The "stems" of the flowers also enter commerce. They possess the odor and taste of the cloves, but they are worth only about one-fifth the price of the cloves, as they deficient in volatile oil. They are largely used as an adulterant in ground cloves, and are used in the manufacture of oil of cloves. In France they are generally known by the name of *griffes de girofle*.

In commerce the varieties of cloves are known by the names of the localities of their growth, and so closely resemble one another as to be distinguished only by experts. The *Penang* and *Amboyna* cloves are the largest and thickest and have been especially prized; the *Bencoolen* cloves from Sumatra are also valuable. During 1952, a total of 1,867,560 pounds of unground cloves was imported into the U. S. A. from British East Africa, Ceylon and Madagascar. In the same year, 456,816 pounds of clove oil entered this country from Madagascar, British East Africa, France and Netherlands.

Description.—*"Unground Clove* is a flower bud from 10 to 17.5 mm. in length, of a dark brown or dusky red color, consisting of a sub-cylindrical, slightly flattened, four-sided hypanthium which contains in its upper portions a 2-celled, inferior ovary with numerous ovules attached to a central placenta, the hypanthium terminated by 4 thick, divergent sepals and surmounted by a dome-shaped corolla, consisting of 4 membranous, imbricated petals, which enclose numerous curved stamens having introrse anthers, and 1 style, at the base of which is a nectar disc. Its odor is strongly aromatic and its taste pungent and aromatic, followed by a slight numbness of the tongue. Clove stems are sub-cylindrical or 4-angled, attaining a length of 25 mm. and a diameter of 4 mm., either simple, branching, or distinctly jointed, and less aromatic than the flower buds.

"Powdered Clove is dark brown. It consists of parenchyma fragments showing the large oval schizolysigenous oil reservoirs, spiral vessels, and a few rather thick-walled, spindle-shaped fibers. Calcium oxalate occurs in rosette aggregates, from 10 to 15 μ in diameter. Fragments of the walls of anthers with characteristic reticulated cells and numerous tetrahedral pollen grains from 15 to 20 μ in diameter are also present. A 50 per cent solution of potassium hydroxide added to a microscope mount of powdered cloves reacts with the volatile oil of the oil reservoirs to form acicular crystals of potassium eugenate." *N.F.*

Standards and Tests.—*Other foreign matter.*—Not over 1 per cent of foreign matter other than stems. *Crude fiber.*—Not over 10 per cent. *Acid-insoluble ash.*—Not over 0.75 per cent. *Clove stems.*—Stone cells, irregular or polygonal, up to about 70 μ in diameter, with thick, porous walls and large lumina, sometimes filled with an orange or yellow amorphous substance, are few or absent. Clove contains not more than 5 per cent of clove stems. *Clove fruit or cereals.*—Starch grains are absent.—*N.F.*

Assay.—This is performed as directed for the official *Volatile Oil Determination. N.F.*

The best cloves exude a small quantity of oil on being pressed or scraped with the nail. When light, soft, wrinkled, pale, and of feeble taste and odor, they are inferior. Those from which the essential oil has been distilled are sometimes fraudulently mixed with the genuine. For monograph on the microscopical structure of cloves, clove stems and clove fruit see Winton, *The Structure and Composition of Foods,* Vol. 3. Powdered cloves sometimes contain an excess of clove stems, and may be adulterated with allspice, wheat middlings and powdered peas or beans. Occasionally clove stems alone are ground and sold as cloves. It is claimed that an enormous quantity of exhausted cloves are dishonestly marketed. The amount of volatile ether extract is the best criterion of the value of cloves.

Constituents.—The most important constituent is a volatile oil (see *Clove Oil*). Trommsdorff found in cloves 18 per cent of volatile oil, 17 per cent of tannin, 13 per cent of gum, 6 per cent of resin, 28 per cent of vegetable fiber, and 18 per cent of water. Peabody (1895) found the percentage of tannin in cloves to range from 10 to 13 per cent, also that it has the same composition as gallotannic acid. Lodibert afterward discovered a green fixed oil, and a tasteless, white, resinous substance which crystallized in silky needles, soluble in ether and boiling alcohol. This substance was called by Bonastre (1827) *caryophyllin.* It is a methylated phenanthrene derivative containing a hydroxyl and a carboxyl group, of the formula $C_{30}H_{48}O_3$, and identical with *oleanolic acid* isolated from a number of plants.

Uses.—Clove is among the most stimulant of the aromatics, but, like others of this class, acts less upon the system at large than on the part to which it is immediately applied. Clove has been administered in the form of powder or as an infusion to relieve nausea and vomiting, correct flatulence, and excite languid digestion.

Dose, 120 to 600 mg. (approximately 2 to 10 grains).

Storage.—Preserve "in well-closed containers and avoid exposure to excessive heat." *N.F.*

Off. Prep.—Compound Lavender Tincture; Aromatic Rhubarb Tincture, *N.F.*

CLOVE OIL. U.S.P., B.P.

Oleum Caryophylli

"Clove Oil is the volatile oil distilled with steam from the dried flower buds of *Eugenia caryophyllata* Thunberg (Fam. *Myrtaceæ*). It contains not less than 85 per cent by volume of total phenolic substances, chiefly eugenol ($C_{10}H_{12}O_2$)." *U.S.P.*

The B.P. recognizes the oil distilled from clove, containing not less than 85.0 and not more than 90.0 per cent of eugenol, $C_{10}H_{12}O_2$.

Oleum Caryophylli Æthereum; Oleum Caryophyllorum; Essentia Caryophilli. *Fr.* Essence de girofle. *Ger.* Nelkenöl. *It.* Essenza di garofani. *Sp.* Esencia de clavo.

For a description of the plant from which this oil is derived see under *Clove*. The oil is obtained by distilling clove with steam, the aqueous phase of the distillate being returned to the still to avoid loss of oil which dissolves in the water. A good quality of clove yields up to about 20 per cent by weight of oil. Most of the oil was formerly brought from Holland or the East Indies, but since the introduction of the cayenne cloves into our markets the reduced price and superior freshness of the drug have rendered the distillation of oil of clove profitable in this country, and the best now sold is of domestic extraction.

Description.—"Clove Oil is a colorless or pale yellow liquid, becoming darker and thicker by aging or exposure to air, and having the characteristic odor and taste of clove. One volume of Clove Oil dissolves in 2 volumes of 70 per cent alcohol." *U.S.P.*

Standards and Tests.—*Specific gravity.*—Not less than 1.038 and not more than 1.060. *Optical rotation.*—Not more than —1° 30′ in a 100-mm. tube. *Refractive index.*—Not less than 1.5270 and not more than 1.5350, at 20°. *Heavy metals.*—The oil meets the requirements of the test for *Heavy metals in volatile oils. Phenol.*—Shake 1 ml. of oil with 20 ml. of hot water: the water is not more than slightly acid toward blue litmus paper. On cooling the aqueous liquid, filtering it through a wetted paper, and treating the filtrate with 1 drop of ferric chloride T.S. only a transient, grayish-green color, but not a blue or violet color, is produced. *U.S.P.*

Assay.—A 5-ml. portion of oil is heated, in a cassia flask, with a potassium hydroxide solution which converts eugenol, as well as any aceteugenol that may be present, to potassium eugenolate, which is soluble in the aqueous liquid. The portion of the oil which is not eugenol remains insoluble and its volume is determined by bringing it into the graduated neck of the flask. The volume of the oily layer should not exceed 0.75 ml., indicating the presence of not less than 85 per cent by volume of total phenolic substances in the oil. *U.S.P.*

Constituents.—Clove oil contains small amounts of vanillin, methyl alcohol and furfurol, but is mainly composed of the unsaturated phenol eugenol (see *Eugenol*), its acetyl derivative, and a sesquiterpene *caryophyllene*. Eugenol acetylsalicylate has also been reported. The eugenol content is the most important criterion of the quality of clove oil.

The characteristic aromatic odor of clove oil, as distinguished from that of eugenol, is due to *methylamylketone*, $CH_3.CO.C_5H_{11}$, which is present only in minute quantity.

Uses.—By virtue of its local irritant effect clove oil stimulates peristalsis and has frequently been employed in the treatment of flatulent colic. It also possesses some local anesthetic action, being a favorite remedy for toothache; for this purpose a small pledget of cotton is saturated with oil and inserted into the carious cavity. It is a powerful germicide, about eight times as strong as phenol, but is not frequently used, except by dentists, because of its irritant properties. Eugenol, the principal constituent of clove oil, has been used internally in daily doses of 3 ml. (approximately 45 minims) as an antiseptic antipyretic; it has also been used in treating patients with gastric or duodenal ulcers by instillation into the stomach (see under *Eugenol* for detailed information). Little, however, is known of its physiological action. According to Leubuscher (*Wien. med. Bl.*, 1889), it is a feeble local anesthetic. Landis (*Therap. Gaz.*, 1909, 33, 386) used clove oil as a stimulant expectorant in tuberculosis and bronchiectasis with good results.

Dose, from 0.12 to 0.4 ml. (approximately 2 to 6 minims).

Storage.—Preserve "in well-filled, tight containers and avoid exposure to excessive heat." *U.S.P.*

Off. Prep.—Diphenhydramine Hydrochloride Elixir, *U.S.P.*; Compound Cardamom Spirit; Aromatic Castor Oil; Aromatic Eriodictyon Syrup; N.F. Toothache Drops, *N.F.*

COBALAMIN CONCENTRATE. N.F.

Vitamin B_{12} Activity Concentrate

"Cobalamin Concentrate consists of the dried, partially purified product resulting from the growth of selected *Streptomyces* cultures or other cobalamin-producing microorganisms. It may contain harmless diluents and stabilizing agents. Cobalamin Concentrate contains in each Gm. not less than 500 mcg. of Cobalamin activity." *N.F.*

Cobalamin concentrate contains cyanocobalamin (vitamin B_{12}) and/or closely related cobalamins and represents a less purified form of the vitamin suitable for many oral formulations; since it is not carried through the purification procedures

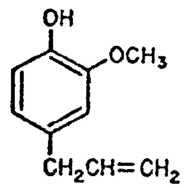
429) found the mydriatic power of eucatropine to be slightly greater than that of cocaine.

A 2 per cent solution is instilled into the conjunctival sac. The maximum amount of the drug thus applied is usually 2 or 3 drops of a 10 per cent solution, which may be repeated once in 5 minutes.

Storage.—Preserve "in tight, light-resistant containers." *U.S.P.*

EUGENOL. U.S.P.

[Eugenol]



"Eugenol is obtained from clove oil and from other sources." *U.S.P.*

Eugenol, the main constituent of clove oil, may be obtained from the latter by treatment with a solution of sodium hydroxide, which converts the eugenol to sodium eugenolate, soluble in the alkaline solution; this solution is separated from the residual oil, treated with acid to liberate free eugenol, which is subsequently washed and distilled in steam or under vacuum. Eugenol is also found in pimenta, cinnamon leaves, sassafras, canella, bay and other plants.

Eugenol is 2-methoxy-4-allylphenol or 3-methoxy-4-hydroxy-allylbenzene, and is of chemical interest as the starting compound in a synthesis of vanillin. It is first converted to iso-eugenol (in which the isomeric alpha-propenyl group replaces the allyl of eugenol) by heating with alkali, after which it is acetylized and oxidized. Iso-eugenol also occurs naturally in several oils, including clove, nutmeg and ylang-ylang.

Description.—"Eugenol is a colorless, or pale yellow liquid, having a strongly aromatic odor of clove and a pungent, spicy taste. Exposure to air causes it to become darker and thicker. Eugenol is optically inactive. Eugenol is slightly soluble in water. It is miscible with alcohol, with chloroform, with ether, and with fixed oils. One volume of Eugenol dissolves in 2 volumes of 70 per cent alcohol. The specific gravity of Eugenol is not less than 1.064 and not more than 1.070." *U.S.P.*

Standards and Tests.—*Boiling range.*—Eugenol distills between 250° and 255°. *Refractive index.*—Not less than 1.5400 and not more than 1.5420 at 20°. *Hydrocarbons.*—A clear mixture, which may become turbid on exposure to air, results on mixing 18 ml. of water with a solution of 1 ml. of eugenol in 20 ml. of 0.5 *N* sodium hydroxide. *Phenol.*—A transient grayish green, but not a blue or violet, color is produced on adding 1 drop of ferric chloride T.S. to 5 ml. of the filtrate from a mixture of 1 ml. of eugenol with 20 ml. of water. *U.S.P.*

The B.P.C. refractive index range (20°) for eugenol is 1.540 to 1.542. The identification tests for eugenol require formation of a blue color with ferric chloride in alcohol solution, and development of an odor of vanillin on heating with alkaline potassium permanganate solution.

Uses.—The medicinal properties and uses of eugenol are practically the same as those of clove oil (*q.v.*). Eugenol appears to be slightly less active as an antiseptic than the natural oil. Eugenol is used by dentists for disinfecting root-canals, as a local anodyne for the relief of hypersensitive dentine and pain and irritation incident to hyperemic and inflamed vital pulps, and as a component of the *zinc-eugenol cement* employed as a temporary filling for carious teeth.

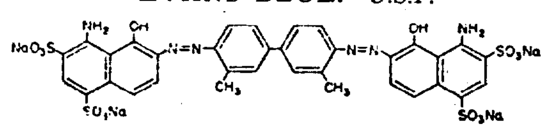
Eugenol has been used to treat patients with gastric or duodenal ulcers by instilling it into the stomach in doses of 0.12 Gm. per Kg. of body weight; after 15 minutes the eugenol was aspirated as completely as possible. The course of treatment consisted of two such applications each week for three weeks. Of 15 patients thus treated, complete relief was obtained in 6 patients, partial relief in 5, and no relief in 4. The period of complete relief persisted for 12, 19 and 21 months, respectively in 3 patients (Bandes *et al., Gastroenterology*, 1951, 18, 391). Eugenol has also been used internally as an antiseptic antipyretic, in doses of 3 ml. daily.

Dose, 0.12 to 0.3 ml. (approximately 2 to 5 minims). Up to 3 ml. (approximately 45 minims) has been given in a period of 24 hours. It is usually used topically.

Storage.—Preserve "in tight, light-resistant containers." *U.S.P.*

Off. Prep.—Zinc Compounds and Eugenol Cement, *N.F.*

EVANS BLUE. U.S.P.



"Evans Blue contains not less than 95 per cent and not more than 105 per cent of $C_{34}H_{24}N_6Na_4O_{14}S_4$, calculated on the dried basis." *U.S.P.*

Azovan Blue, *B.P.C.* T-1824.

This diazo dye, the tetrasodium salt of 4,4′-bis-[7-(1-amino-8-hydroxy-2,4-disulfo)naphthylazo]-3,3′-bitolyl, may be prepared by coupling of 1 mole of diazotized *o*-tolidine with 2 moles of 1-amino-8-naphthol-2,4-disulfonic acid.

Description.—"Evans Blue is a green, bluish green, or brown powder. It is odorless. The dried product is hygroscopic. Evans Blue is very soluble in water. It is very slightly soluble in alcohol and practically insoluble in benzene, in carbon tetrachloride, in ether, and in chloroform." *U.S.P.*

Standards and Tests.—*Identification.*—(1) On adding sodium nitroferricyanide T.S. to a solution of a sodium fusion of the dye a red-violet color appears, indicating the presence of sulfide. On adding ferrous sulfate and ferric chloride to another portion of the same solution a blue color appears, indicating the presence of cyanide. (2) A 0.00035 per cent solution of Evans

Original Reports

Anthelmintic action of constituents of vegetal essences

by MM. G. Valette, R. Cavier and Mrs. J. Debelmas (*)

(*) Presented at the Academy of Pharmacy, session of July 1, 1953.

Research conducted by two of our group (3) on the anthelmintic action of vegetal essences has shown that aside from reputed vermifugal products such as essences of Absinthe, of Chenopode and of Thyme, other essences proved to be very active vermicides, in vitro as well as in the infested animal. In paeticular the activity of essences of Cinnamon and Clove on Nematodes and Cestodes of the Mouse proved far superior to the above mentioned essences.

We were naturally led to resume the experiments, no longer with essential oils, but with their chemically defined constituents, or products of similar composition, and this in the hope of bringing some new elements to the notions acquired on the relationship between chemical composition and anthelmintic activity.

TECHNIQUE

The pharmacological tests to which we resorted for this study are the "in vitro" and "in vivo" tests which are described in the recent work of R. CAVIER (2) and whi h are in current use in most pharmacodynamics laboratories.

1. In vitro test on a coproculture of Rhabditis macrocerca, Kreis and Faust (1933), sacrophyte Rhabditidy isolated from the stools of the wild rabbit and particularly resistant to the action of anthelmintics (5).

2. In vitro test on whole Ascaris suum Goeze (1882) (LAMSON and BROWN /7/.

3. In vitro test on the anterior segment of the same Ascaris (TOSCANO, RICO REBELLO, DA COSTA (10), BALDWIN (1)).

In these in vitro tests our results are expressed by the time necessary to obtain the death of the animal, or of all the animals.

4. In vivo tests on the Oxyuridates of the white Mouse, Scyphacia obvelata Rudolphi (1802) and Aspicularis tetraptera Nitzsch (1821), according to the

DESCHIENS technique, in which account is taken of the percentage of disinfested animals, the substance having been administered to a lot of at least ten animals

In fact, to judge the value of a new anthelmintic, one cannot limit oneself to a single type of test and the results obtained in vitro merely serve for a rough sorting out of substances presumed active. This study must necessarily be continued on the infested animal in order to take account of the toxicity of the substance for the host as well as of the influence of the environment on the behavior of the parasite and on the anthelmintic activity to be studied.

Our study involved mainly products representing the following four chemical series:

Cinnamic derivatives - aromatic aldehydes - alkylphenols - terpenic derivatives.

All these substances were used in the formof aqueous emulsions obtained by means of gum arabic and brought to a concentration of M/100. For some of the more active products it was necessary to resort to stronger dilutions of 3 M/1 000 or 5 M/1 000.

Our report is limited to substances which we felt presented the greatest interest.

RESULTS

1. Cinnamic series. - Cinnamic aldehyde proves clearly more anthelmintic than the corresponding alcohol, while cinnamic acid is devoid of any activity. Ethyl and benzyl esters occupy an intermediate position between the aldehyde and the alcohol. All these products are little toxic.

2. Aromatic aldehyde series. - Cinnamic aldehyde takes the first rank; immediately after come salicylic aldehyde; but a toxic effect for the Mouse appears with this substance.

The anisic aldehyde proves slightly less active and just as toxic.

The benzoic aldehyde is yet less effective in vitro and in vivo.

As to vanillin, it has no activity at all.

3. Alkyl-phenol series. - In confirmation of the results previously obtained with the essences of Cinnamon and Clove, eugenol proves as active in

vitro and in vivo as cinnamic aldehyde; immediately after and in the order indicated come: isoeugenol, isosafrol and safrol. Anethol is clearly less active. One should furthermore point out a fairly high toxicity in the case of eugenol and of anethol.

The anthelmintic action of thymol is well known and commonly used to good effect; however its isomer, carvacrol, proved clearly more active in vitro as well as in vivo, contrarily to the conclusions brought out by KOCHMANN (6). It should be noted in addition that these two subseances are fairly toxic for the Mouse. Our experiments proved in addition that menthol is much less anthelmintic than thymol, the corresponding unsaturated derivative, while TANABE (9), on the basis of in vitro tests on Ascaris of the Hog, had observed an equal activity for the two bodies.

4. Terpenes and derivatives series.- It is difficult to establish a classification for the three terpenic carbides studied. According to in vitro tests on the Ascaris, limonene proves the most active; then follow pinene alpha, then phellandrene. In the infested Mouse, on the contrary, the activity is maximum in the case of pinene alpha, smaller in the case of phellandrene and minimum in the case of limonene. These three carbides are relatively toxic for the Mouse.

Geraniol, citronellol and citral prove approximately as anthelmintic as pinene alpha; geraniol, devoid of toxicity in the Mouse, takes the first rank according to the results of in vitro tests on Rhabditis.

The body of our results appear in the preceding table.

DISCUSSION

The different pharmacodynamic tests used during these tests on the anthelmintic properties of constituents of vegetal essences gave us results having a satisfactory degree of agreement: with the exception of the terpenic carbides studied the most active substances in vivo generally prove the most active in vitro and the two tests performed in vitro respectively on Rhabditidates and on Ascaris lead to similar conclusions.

Of all these substances we have passed in review we can consider the following as possessed of unquestionable activity on the Nematodes, and in decreasing order: carvacrol, eugenol, isoeugenol, cinnamic aldehyde, salicylic aldehyde, pinene alpha and geraniol. And among these subtances we must mention particularly as completely devoid of toxicity: isoeugenol, cinnamic aldehyde and geraniol.

From these facts a certain number of conclusions can be drawn concerning the influence of the structural characters on anthelmintic acitivity:

1. The aldehyde function appears favorable to the pharmacodynamic action studied, at least in the case of the cinnamic series;

2. The presence of an unsaturated chain on the benzenic nucleus increases the activity (passing from the little active benzoic aldehyde to the very active cinnamic aldehyde);

3. The presence of a free phenol function increases the anthelmintic activity, but also the toxicity (passing from the benzoic aldehyde to the salicylic aldehyde, or from the anisic aldehyde to the salicylic aldehyde);

4. Hydrogenation of the benzenic nucleus decreases the anthelmintic power (passing from the very active thymol to the almost inactive menthol).

SUMMARY

1. The anthelmintic action of a certain number of constituents of the vegetal essences has been studied on the Nematodes through in vitro and in vivo tests.

2. Carvacrol, eugenol, isoeugenol, cinnamic aldehyde, salicylic aldehyde, pinene alpha and geraniol prove to be the most active of the derivatives studied.

3. The influence of the chemical composition on the appearance of anthelmintic properties is discussed.

A detailed report on this research can be found in the work of Mrs. J. DEHELMAS (4).

(Faculty of Pharmacy of Paris, Pharmacodynamics and Applied Zoology Laboratores).

TABLE - Compared Anthelmintic action and toxicity of various constitutents of vegetal essences.

	Molecular concentration	In vivo test; percentage of disinfested animals	Toxicity; percentage of animals killed	In vitro test; on Rhabditis; time of death in minutes	In vitro test; on Ascaris; time of death
1. Series cinnamic:					
Aldehyde cinnamic	10^{-2}	100	0	5	1 h. 30
Cinnamate d'ethyle	"	60	0	15	6
2. Aldehydes:					
Aldehyde cinnamic	"	100	0	5	1 h. 30
Aldehyde salicylic	"	100	25	10	3
Aldehyde anisic	"	100	25	60	10
3. Alkylphenols:					
Eugenol	"	100	50	5	45 min.
Isoeugenol	"	100	0	"	2
Safrol	"	100	0	10	8
Isosafrol	"	100	10	10	4 h. 30
Anethol	"	100	20	15	7
Carvacrol	$3\ 10^{-3}$	100	20	15	45 min.
Thymol	"	10	20	15	1 h. 45
4. Derivates of Terpenes					
Pinene	10^{-2}	100	33	"	1
Phellandrene	"	85	20	"	13
Limonene	"	40	29	"	30 min.
Geraniol	"	100	0	8	1 h 30
Citronellol	"	100	20	25	1 h 30
Citral	"	100	33	20	1

Translated by Carl Demrick Associates, Inc./ARB/db

Coniferyl Aldehyde as a Constituent of Oils Containing Eugenol

By SHOUKRY KHALIL WAHBA* and JOSEPH E. SINSHEIMER

The presence of coniferyl aldehyde in eugenol, clove oil, myrica oil, pimenta oil, and sassafras oil has been demonstrated. The characteristic cherry red developed by coniferyl aldehyde with phloroglucinol and hydrochloric acid can be used for a colorimetric assay of coniferyl aldehyde and contributes a significant portion of the color developed by eugenol-containing oils with these reagents.

A CHARACTERISTIC cherry red is produced when a phloroglucinol-hydrochloric acid reagent is allowed to react with clove oil (1). Eugenol has been considered responsible for this test (2, 3), and since many allyl compounds give a positive test, it has been suggested as a functional group test for this moiety (2).

Adler (4), however, has explained the positive phloroglucinol reaction of lignin and its prototype compounds upon the formation of coniferyl aldehyde or related compounds. He was able to isolate the 2,4-dinitrophenylhydrazone of methylconiferyl aldehyde from methyleugenol and to demonstrate that a purified sample of eugenol does not give a positive phloroglucinol test.

Although coniferyl aldehyde has not been listed as a constituent of *Eugenia caryophyllata* (5–7), it appears that it may be present in this plant and other eugenol-containing species as well as oils obtained from such plants. This investigation was undertaken to detect the presence of coniferyl aldehyde in eugenol-containing oils and to isolate the compound from eugenol U.S.P.

EXPERIMENTAL

Apparatus and Materials.—Spectrophotometric determinations were made with Beckman model DB, Zeiss PMQ II, and Perkin-Elmer model 137 spectrophotometers. All melting points were made on a Kofler hot stage and are corrected. Samples of eugenol, clove oil, myrica oil, pimenta oil, and sassafras oil met the present or former requirements for official purity. All chemicals were of reagent grade and were used without further purification.

Chromatographic Procedures.—One-tenth of a milliliter of alcoholic solutions containing 2% clove oil, myrica oil, pimenta oil, or eugenol; 0.01% coniferyl aldehyde, or 0.2% vanillin were chromatographed as suggested by Higuchi (9) on Whatman No. 1 filter paper, using the aqueous phase of a mixture of water:petroleum ether (90–100°):benzene:methanol (50:50:50:1) or butanol saturated with 3% aqueous ammonia.

Presence of vanillin and coniferyl aldehyde was detected by spraying with a 0.5% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid or with a 1% solution of phloroglucinol in a mixture of 10 ml. of hydrochloric acid and 90 ml. of alcohol.

Since sassafras oil has a low concentration of eugenol, a 0.5-ml. alcoholic solution containing 50% of this oil was chromatographed as a band on a 23 × 57-cm. sheet of Whatman No. 1 paper using the butanol-ammonia system. The zone corresponding in R_f to coniferyl aldehyde was extracted with alcohol and the concentrated extract rechromatographed as a single spot with the butanol-ammonia system. Coniferyl aldehyde was then detected with the phloroglucinol spray.

Isolation of Coniferyl Aldehyde.—The 2-Gm. brown residue remaining after distillation up to 250° of the major portion of 200 ml. of eugenol was dissolved in 5 ml. of ethanol. One-milliliter portions were chromatographed as bands on 23 × 57-cm. sheets of Whatman No. 1 filter paper using the aqueous system described above in a descending technique.

The coniferyl aldehyde zone was located with the aid of the phloroglucinol spray on a portion of this paper. This zone was extracted with alcohol, the solution concentrated to 1 ml. under reduced pressure, and rechromatographed in the same manner. The coniferyl aldehyde zone was then extracted with boiling benzene and the extract concentrated to 1 ml. Ten milligrams of yellow needles were isolated after cooling the solution overnight.

Colorimetric Determination of Coniferyl Aldehyde.—To a 2-ml. solution of from 1 to 6 mcg. of coniferyl aldehyde in acetone was added 1 ml. of a 0.2% solution of phloroglucinol in a mixture of 5 ml. of hydrochloric acid and 95 ml. of acetone. The cherry red which develops must be measured at 540 mμ within 10 to 15 minutes after the start of the reaction.

Rate of Formation of Coniferyl Aldehyde.—A mixture of 200 ml. of eugenol and 20 ml. of a 1% alcoholic solution of 2,4-dinitrophenylhydrazine was distilled under nitrogen. The fraction collected at 248° was tested at a concentration of 20 mg. in 2 ml. of acetone as outlined under the *Colorimetric Determination of Coniferyl Aldehyde.* Determinations were made immediately following distillation and at intervals up to 48 hours after the distillation.

RESULTS AND DISCUSSION

It was not only possible to indicate the presence of coniferyl aldehyde by paper chromatography but also to isolate this aldehyde from eugenol U.S.P. The residue after distillation of the major portion of a sample of eugenol was chromatographed on paper using the aqueous phase of the system suggested by Higuchi (9) for the separation of coniferyl aldehyde in his lignin studies. While the presence of two carbonyl compounds was indicated through the use of a 2,4-dinitrophenylhydrazine spray, only one of the compounds gave a cherry red test with a phloroglucinol-hydrochloric acid spray. The red produced by this latter spray was used to locate the coniferyl aldehyde zone. Extractions of this zone yield crystals melting at 78° which did not show a depression when mixed with an authentic sample of coniferyl aldehyde.[1] A 2,4-dinitrophenylhydrazone was also prepared from the isolated material and melted at 246–248°. A mixture of this hydra-

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[1] The authors are indebted to Dr. John C. Pew, Forest Products Laboratory, Madison, Wis., for generously furnishing us with a reference sample of coniferyl aldehyde.

TABLE I.—CHROMATOGRAPHIC CHARACTERISTICS OF CONIFERYL ALDEHYDE AND VANILLIN

Compd.	R_f Values[a] System I[b]	System II[c]	Phloroglucinol Color Reaction
Coniferyl aldehyde			
Reference	0.61	0.58	Cherry red
Isolated	0.60	0.58	Cherry red
Vanillin			
Reference	0.68	0.46	Pale yellow
Isolated	0.67	0.46	Pale yellow

[a] These values are for Whatman No. 1 paper at 21° and are averages with a standard deviation of less than .015. [b] The aqueous phase of a mixture of water: petroleum ether (90–100°): benzene: methanol (50:50:50:1). [c] Butanol saturated with 3% aqueous ammonia.

zone and one prepared from the authentic sample showed no depression in melting point. The infrared spectra of both the aldehyde and its hydrazone were identical with the reference materials.

The chromatographic characteristics of the isolated and reference coniferyl aldehyde are summarized in Table I. The second aldehyde present in the sample of eugenol is consistent in its chromatographic characteristics with those of vanillin as also summarized in Table I. For both coniferyl aldehyde and vanillin, a mixture of the isolated and reference aldehyde chromatographed as a single spot with characteristic R_f values. It is evident that coniferyl aldehyde can be separated from any vanillin present.

In addition to the isolation of coniferyl aldehyde from eugenol, the presence of this aldehyde in eugenol-containing oils was demonstrated by paper chromatography. When clove, myrica, and pimenta oils were chromatographed on paper using the butanol-ammonia system, a characteristic red developed with the phloroglucinol-hydrochloric acid spray and had the same R_f value as the reference coniferyl aldehyde.

The presence of coniferyl aldehyde can even be demonstrated for sassafras oil which contains eugenol as a minor constituent in the order of 0.5% (8). Thus, sassafras oil yields a chromatogram with the characteristic color and R_f value for coniferyl aldehyde but requires a preliminary concentration of the coniferyl aldehyde.

Coniferyl aldehyde can be determined colorimetrically with phloroglucinol under a variety of conditions. In this investigation, maximum stability was obtained with acetone as a solvent and by limiting the amount of hydrochloric acid used. For a concentration of 6 mcg. in 3 ml. of the reaction mixture, a maximum absorbance of 0.485 was obtained after 10 minutes and was stable for another 5 minutes. The color developed during this period was proportional to concentration up to 6 mcg. in 3 ml. of the reaction mixture. However, it should be noted that although coniferyl aldehyde contributes significantly to the color developed by eugenol-containing oils upon treatment with phloroglucinol and hydrochloric acid, it is not advisable to use this reaction for the direct determination of coniferyl aldehyde in these oils. Other constituents produce color under the same conditions. For example, vanillin produces a pale yellow, while safrol yields an intense red.

A positive cherry red phloroglucinol test on freshly pressed oil and clove buds demonstrated that the formation of coniferyl aldehyde also occurs at this point and is not an artifact due to processing of the oil or to the method of isolation used in this investigation. It becomes evident that coniferyl aldehyde must be widely present in eugenol preparations when the rapid rate of formation of the compound is noted. Eugenol was distilled under nitrogen and a colorless oil obtained which turns pale yellow after a 1-hour exposure to the atmosphere. A 20-mg. sample of the colorless distilled eugenol failed to give a positive test with phloroglucinol when examined immediately. However, when the distilled eugenol was exposed to atmospheric oxygen, a concentration of 20 mg. in 3 ml. of reaction mixture gave an absorbance of 0.010 after 5 minutes and at the same concentration reached a maximum absorbance of 0.325 after 22 hours of exposure to the atmosphere.

SUMMARY

Coniferyl aldehyde has been isolated from eugenol and shown to be present in eugenol-containing volatile oils.

Coniferyl aldehyde forms rapidly from eugenol and is essentially responsible for the characteristic cherry red color developed by eugenol-containing oils with phloroglucinol and hydrochloric acid.

A colorimetric procedure has been developed for the estimation of coniferyl aldehyde.

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EXPERIMENTAL PEPTIC ULCERATION.

Part IV.

EFFECT OF MUCOUS STIMULANTS ON HISTAMINE-INDUCED ULCERATION.

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(From the Central Drug Research Institute, Lucknow.)

[Received for publication, May 20, 1958.]

In a study of the significance of mucous barrier in the prevention of histamine-induced ulceration, it was suggested that stimulants which may provoke mucous cells to secrete mucus without markedly stimulating peptic or parietal cells and having no ill effects on the mucosa of the stomach, may prove of value in the prevention of experimental peptic ulceration (Zaidi and Mukerji, 1958). An attempt has, therefore, been made to study this aspect.

Mucous stimulants.—Bolton and Goodhart (1933) studied the production of pure mucus from the cat's stomach by using ether or pilocarpine as a stimulatory agent. Babkin, Hebb and Kruegcr (1941) stimulated mucous secretion of the stomach with 1 per cent solution of acetic acid. Tulin *et al.* (1947) showed secretion of gastric mucin following alcohol test meal. Spices and condiments, when administered, provoked the production of mucus (Sanchez-Palomera, 1951; and Zaidi and Mukerji, *loc. cit.*). Hollander *et al.* (1947) observed that 1 per cent emulsion of mustard oil in water was a poor stimulus for gastric mucous secretion, while Hollander and Lauber (1948) noted that 5 per cent eugenol, a major component of clove oil, was effective. Sonnenblick *et al.* (1950) observed that repeated topical application of eugenol in high concentration to the mucosa of the Heidenhain pouch contained desquamated epithelial cells including columnar, parietal and mucous neck cells in the early stages of application, but after 6 or 7 cycles of stimulation the stage of mucous barrier normally protecting the underlying epithelium was completely destroyed which, however, rapidly recovered. Lauber and Hollander (1950) showed that the intra-gastric instillation of eugenol in low concentration (0·2 g./kg.) was safe for dogs even when repeated. With these observations in view, alkali salts of eugenol were prepared so that when administered into the stomach they would split and liberate very small amounts of eugenol just enough to stimulate mucous secretion and form a mucous barrier without causing desquamation. The liberated alkali would be expected to neutralize the excess free acid.

Material and method.

Animals.—Male guinea-pigs (average weight 500 g.) of the Institute colony were used.

Preparation of compounds:

Sodium eugenate ($C_{10}H_{11}O_2Na$).—This was prepared by a modified method of Scheuch (1863) by reacting equimolar quantities of sodium methoxide, dissolved in minimum amount of methanol, and eugenol in dry benzene. Sodium eugenate separated out as a white precipitate which was filtered off, washed well with benzene and light petroleum and dried in vacuo. The salt is completely and readily soluble in water.

Barium eugenate ($C_{20}H_{22}O_4Ba$) was prepared according to the method of Williams (1858) by warming of eugenol with baryta water. It was re-crystallized from water and alcohol and was obtained in glistening plates. The salt is partially soluble in water.

Calcium eugenate ($C_{20}H_{22}O_4Ca$) was likewise obtained by treating eugenol with a staturated aqueous solution of calcium hydroxide. Its solubility in water is 0·3 per cent.

Production of peptic ulceration.—Acute peptic ulceration was produced with massive doses of histamine (50 mg.) in guinea-pigs protected with an anti-histaminic-promethazine hydrochloride by the method of Halpern and Martin (1946) modified by Zaidi and Mukerji (*loc. cit.*).

Production of mucous barrier.—Mucous barrier was produced with the administration of above mucous stimulants by the method described previously (Zaidi and Mukerji, *loc. cit.*).

PLAN OF THE EXPERIMENT.

The animals were divided into six groups and in each histopathological investigations of the gastric mucosa and biochemical studies of the gastric juice were made. Group I included 10 animals, groups II, III and V included 11 animals each, while groups IV and VI had 14 animals. The test meal consisted of water in group I, 1 per cent eugenol in group II, 0·5 per cent eugenol in group III and sodium, barium and calcium eugenate in water in groups IV, V and VI, respectively, corresponding to 0·5 per cent free eugenol.

Histopathological technique.—Autopsy was performed on all the animals which died during the experimental period and on the rest which were sacrificed 4 hours after the injection of histamine. Naked-eye examination of the stomach for motility, distension, congestion and perforation was made. The stomachs were incised, gastric contents collected and the degree of ulceration graded, maximum score being hundred (Zaidi and Mukerji, *loc. cit.*). The index of ulceration was calculated according to the method of Pauls, Wick and McKay (1947). Tissue blocks fixed in formol saline were cut, and paraffin sections stained with hæmatoxylin and eosin and with PAS-stain for mucin (Pearse, 1949).

Biochemical technique.—The gastric contents from each animal were centrifuged to remove food particles. Free and total acidity was then titrated with 0·01 N-NaOH using Topfer's and phenolphthalein reagents and expressed as m.eq./l and peptic activity determined by Mett-tube method.

Results.

Biochemical determinations.—Appreciable differences in the volume of gastric juice were not noted in any of the groups. The mean values ranged from 4·4 c.c. to 6·5 c.c. Marked differences in the free acidity were observed. Minimum free acidity was noticed with barium eugenate followed by 1 per cent eugenol, while the values with 0·5 per cent eugenol, sodium eugenate and calcium eugenate ranged within narrow limits. Similar changes were observed in all the groups in respect of total acidity. Eugenol and its salts, used in the present experiments, showed a tendency to suppress the peptic activity. Mucin was stimulated most by 1 per cent eugenol, while with 0·5 per cent eugenol the values were correspondingly low. Calcium eugenate stimulated mucus of nearly the same order as 1 per cent eugenol (Table I and Text-figure).

Table I.

Summary of results of investigation of gastric content.

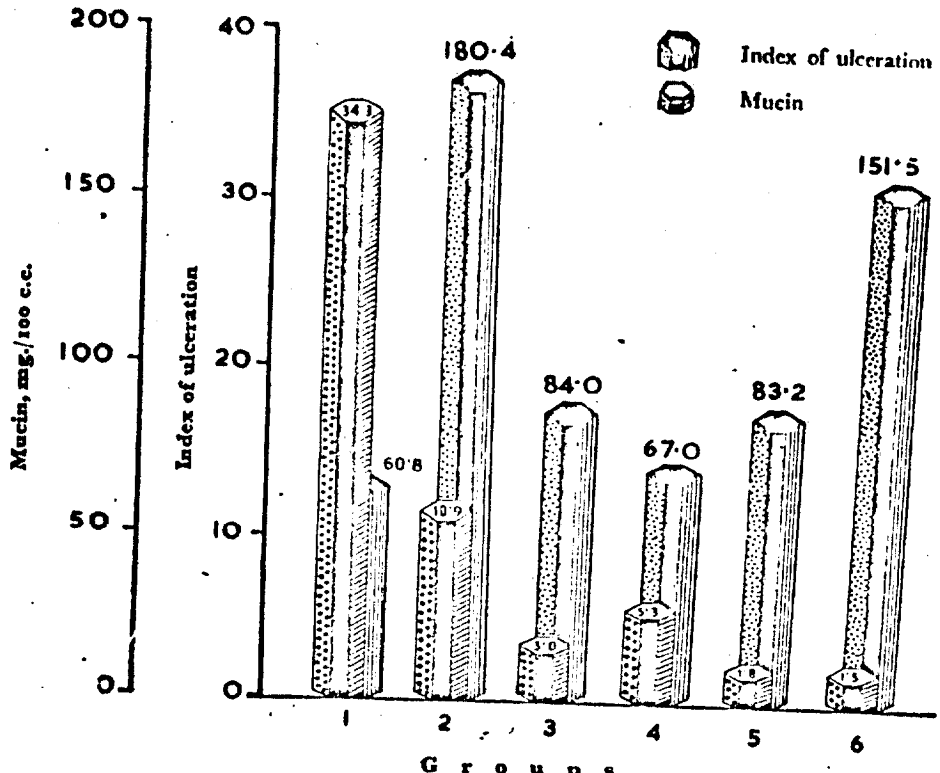
Group:		I	II	III	IV	V	VI
Number of animals		10	11	11	14	11	14
Volume, c.c.	Mean	5·9	5·2	4·7	6·5	6·2	4·4
	Median	5	5	4	6	7	3·5
	Range	2·3–8·3	1·7–10·7	1·1–7·8	1·7–10·0	2·0–11·0	1·3–7·7
Free acidity, m.eq./l	Mean	43·4	10·0	22·3	20·6	10·4	22·0
	Median	40	4	20	13	0	20
	Range	0–92	0–46	0–56	0–68	0–4	0–50
Total acidity, m.eq./l	Mean	59·1	22·8	45·1	38·4	12·9	41·0
	Median	43	20	40	40	12	40
	Range	5–114	10–52	16–70	2–80	8–22	8–68
Peptic activity Mett unit	Mean	15·5	1·0	0·0	0·3	0·0	0·0
	Median	1	0	0	0	0	0
	Range	0–92	0–8	0–0	0–2	0–0	0–0
Mucin, mg. per cent	Mean	60·8	180·4	84·0	67·0	83·2	151·5
	Median	47	168	80	64	74	147
	Range	21–147	78–494	42–147	42–158	40–258	105–252

Group I histamine and promethazine hydrochloride, group II 1·0 per cent eugenol, group III 0·5 per cent eugenol, group IV sodium eugenate, group V barium eugenate and group VI calcium eugenate (ulceration was produced in groups II to VI by the same procedure as in group I).

Histopathology:

Gross appearance.—The naked-eye examination of the stomach *in situ* in groups I to VI showed in some animals ulceration of varying degree and extent in fundus and pylorus, while the mucosa of unulcerated animals had normal appearance. In groups II to VI visible mucous was present in some animals in the gastric juice and also formed thin layer on the gastric mucosa and this was absent in group I. The degree and index of ulceration is shown in Table II and the Text-figure.

TEXT-FIGURE.



Group I histamine and promethazine hydrochloride
Group II 1·0 per cent eugenol
Group III 0·5 per cent eugenol
Group IV sodium eugenate
Group V barium eugenate
Group VI calcium eugenate

TABLE II.

Summary of results of the mean degree of gastric ulceration.

Group.	Degree of ulceration:		Index of ulceration:	
	Fundus.	Pylorus.	Fundus.	Pylorus.
I	38·1	1·4	34·3	0·6
II	17·2	2·7	10·9	1·5
III	6·5	1·0	3·0	0·2
IV	10·7	1·1	5·3	0·3
V	5·0	0·5	1·8	0·1
VI	3·6	0·7	1·5	0·1

Group I histamine and promethazine hydrochloride, group II 1·0 per cent eugenol, group III 0·5 per cent eugenol, group IV sodium eugenate, group V barium eugenate and group VI calcium eugenate (ulceration was produced in groups II to VI by the same procedure as in group I).

$$\text{Index of ulceration} = \frac{\text{Degree of ulceration} \times \text{per cent of group ulcerated}}{100}$$

Microscopic appearances.—Microscopic studies of the animals of group I (control) showed ulceration of varying degree. Six out of ten animals had deep ulceration, three superficial ulceration and hæmorrhage and one had no ulceration. In ulcerated areas marked congestion, œdema of submucosa and infiltration with acute inflammatory cells mostly polymorphonuclear leucocytes was present. Mucous cells were strongly positive with PAS-stain. One animal which had no ulceration showed depletion of mucin from mucous cells. Desquamation of the gastric mucosa was not noted in non-ulcerated area in any of the animals.

When 1·0 per cent eugenol was administered (group II), out of eleven animals four showed complete protection from ulceration. One animal had deep and six superficial ulceration and hæmorrhage. Besides, the ulcerated animals showed generalized desquamation of gastric mucosa. Mucous cells were shrunken and PAS-negative except in one animal which had partial discharge of mucin. Slight œdema and acute inflammatory cells were present in the submucosa of all the animals. The administration of 0·5 per cent eugenol (group III) showed comparatively more protection from ulceration than 1·0 per cent eugenol. There was absence of deep ulceration. Five out of eleven animals showed only superficial ulceration. No evidence of œdema or acute inflammatory reaction was seen. Most of the animals showed partial discharge of mucin from mucous cells. The desquamation in the non-ulcerated area of the gastric mucosa was noted only in two animals.

With sodium eugenate (group IV) the reaction of gastric mucosa was similar to that with 0·5 per cent eugenol except that one animal had deep ulceration and one showed desquamation of the non-ulcerated area. Barium eugenate (group V) showed minute superficial erosions in four animals. Desquamation, œdema or inflammatory reaction was absent and most of the animals had partially discharged mucin as evidenced by PAS-stain. With calcium eugenate (group VI) eight animals showed complete protection from ulceration. The submucosa in these animals did not show evidence of œdema or inflammatory reaction. The mucous cells were shrunken and had partially depleted mucin. Two animals showed superficial ulceration with slight inflammatory reaction and the mucosa in the non-ulcerated area did not show any desquamation.

Discussion.

The present experiments show that eugenol has a marked action in the prevention of histamine-induced ulceration. Our experiments also demonstrate that it lowered free acidity and peptic activity and increased mucous secretion. Eugenol, when used in 0·5 per cent emulsion, prevented mucosal damage by a significant mucous response. In higher concentration of 1·0 per cent, eugenol did not prevent histamine-induced ulceration to the same degree as 0·5 per cent, although the mucous secretion was higher and the free acidity lower. The comparative increase in ulceration may be due to intense irritant effect of 1·0 per cent eugenol on the gastric mucosa. This is borne out by our experiments where marked desquamation of gastric mucosa, slight œdema of submucosa, acute inflammatory reaction and complete exhaustion of mucous cells possibly rendered mucous barrier inefficient to prevent ulceration.

Studies with the alkali salts of eugenol showed that sodium eugenate brought similar changes in gastric mucosa and juice as 0·5 per cent eugenol. Barium eugenate, on the other hand, had marked preventive action against histamine induced peptic ulceration. Free acidity was markedly lowered and peptic activity inhibited, while mucous production was of fair degree. This inhibition of free acidity and peptic activity may be due to the direct action of barium chloride, produced by the splitting of barium eugenate under the influence of the free acid. Further, barium chloride may have constricted the gastric arterioles and under this state of gastric mucosa histamine possibly could not reach the parietal cells to produce excessive amount of acid, cause œdema and ulceration. Goodman and Gilman (1949) noted that barium chloride in high doses paralysed the central nervous system and produced gastro-intestinal disturbances. In our experiments barium chloride formed in the stomach was so small that it did not cause any visible toxic reaction.

Kraus and Hollander (1949) noted that topical application, to dog's Heidenhain pouches, of buffered 1 per cent eugenol emulsion containing calcium exercised the same mucigogue and desquamatory action as did control emulsion containing no added calcium. The protection from histamine-induced ulceration in our experiments with calcium eugenate was marked which appears to be better than the other compounds used. This may be due to the splitting of calcium from eugenol, whereby eugenol stimulated mucous secretion and calcium combined with hydrochloric acid to form calcium chloride. Grant (1941) showed that calcium chloride inhibited both the nervous and chemical types of gastric secretion and Cook and Martin (1948) further observed that it was antispasmodic. In the present investigation calcium eugenate inhibited free acidity as compared to control group, where no mucous secretion was stimulated, but how far this inhibition was due to the formation of calcium chloride is difficult to assess.

Fogelson (1931) reported that powdered gastric mucin obtained from hog's stomach was of therapeutic value in the treatment of peptic ulcer and Kim and Ivy (1931) also noted that mucin coated the surface of the ulcer, protected it against peptic digestion and neutralized hydrochloric acid. Rivers, Vanzant and Essex (1932), on the other hand, noted that mucin was secretogogue, like histamine, and that it produced gummy, sticky mass in the stomach and hence was of doubtful value in the treatment of peptic ulceration. Recently, Spira (1956) observed that the results of mucin therapy were not encouraging. It is suggested that the failure of mucin therapy may be the inability of mucin to reach the parietal cells lying in the gastric pits. In the presence of high acidity mucin may become more viscous, adhere to the superficial surface and clog the gastric pits. It may thus be unable to neutralize the acidity at the site of its production. These factors have been avoided by the direct stimulation of mucous cells. In the present experiments, the stimulation of the mucous cells produced mucous which simultaneously neutralized acidity, depressed peptic activity and prevented ulceration.

Our investigations indicate that eugenol and its various salts employed in the present experiments have a value in the prevention of histamine-induced ulceration. The mechanism of prevention becomes significant when they are used in small doses, which give an exaggerated physiological response 'a true reflexe defense' in the form of mucous which is utilized to neutralize hyperacidity, depress peptic activity and prevent experimental ulceration.

SUMMARY.

1. Experimental acute peptic ulceration was produced with massive doses of histamine in guinea-pigs protected by an antihistaminic, promethazine hydrochloride.

2. Eugenol in small doses (0·5 per cent emulsion) lowered free acidity and peptic activity and gave a significant mucous response which prevented ulceration.

3. Studies with sodium, barium and calcium eugenates, in the prevention of ulceration have been made. These compounds, when administered in the stomach, liberated eugenol which stimulated mucous secretion to form mucous barrier against ulceration, and the alkali neutralized the free acidity. Calcium eugenate appears to be more active in the prevention of ulceration than other compounds used.

4. The significance of mucous stimulants in the prevention of acute peptic ulceration has been discussed.

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